FEB 4 1929

THE JOURNAL

OF

EXPERIMENTAL MEDICINE

EDITED BY

SIMON FLEXNER, M.D. PEYTON ROUS, M.D.

VOLUME XLIX, No. 2 FEBRUARY 1, 1929

Medical Library

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Entered as second-class matter June 3, 1916, at the Post Office at Baltimore, Md., under the act of March 3, 1897.
Accepted for mailing at special rate of postage provided for in section 1103, act of October 3, 1917.
Authorized June 29, 1918.

Made in the United States of America

PUBLICATIONS OF THE ROCKEFELLER INSTITUTE FOR MEDICAL RESEARCH

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*Out of print.

WAVERLY PRESS
BALTIMORE, U. S. A.

THE CHEMICAL NATURE OF THE SUBSTANCES REQUIRED FOR CELL MULTIPLICATION.

II. Action of Glutathione, Hemoglobin, and Ash of Liver on the Growth of Fibroblasts.

By LILLIAN E. BAKER, Ph.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, October 5, 1928.)

For some time it has been known that fibroblasts and epithelial cells proliferate indefinitely *in vitro* in a medium of plasma and embryo juice.[1] If all the factors that function in this unlimited reproduction of tissues could be known, it seems probable that much could be done to accelerate the processes of repair. Such knowledge might also throw light on the problem of development and growth of tumor. With these ideas in mind, a series of investigations was begun, the object being to ascertain the chemical nature of the substances which are utilized by the tissues for maintenance and growth. It was found that the protein fraction[2] of embryo juice was mainly responsible for the multiplication of the cells. All other proteins tested lacked this growth-promoting power, and even the protein of the embryo juice became inactive on purification by repeated precipitation. However, proteoses prepared from many different proteins stimulated enormously the multiplication of fibroblasts and other cells.[3] Therefore, the hypothesis was advanced that enzymes in the embryonic juice under the influence of the acid produced by the cells hydrolyzed the

[1] Carrel, A., *J. Exp. Med.*, 1913, xvii, 14. Fischer, A., *J. Exp. Med.*, 1922, xxxv, 367. Carrel, A., and Ebeling, A. H., *J. Exp. Med.*, 1921, xxxiv, 317; 1923, xxxviii, 487. Carrel, A., *Physiol. Rev.*, 1924, iv, 1; *Compt. rend. Soc. biol.*, 1927, xcvi, 603.

[2] Baker, L. E., and Carrel, A., *J. Exp. Med.*, 1926, xliv, 387; *Compt. rend. Soc. biol.*, 1926, xcv, 157.

[3] Carrel, A., and Baker, L. E., *Proc. Soc. Exp. Biol. and Med.*, 1926, xxiii, 627; *J. Exp. Med.*, 1926, xliv, 503; *Compt. rend. Soc. biol.*, 1926, xcv, 359.

embryo protein to proteoses which promoted growth. The products of the further hydrolysis of proteins, *i.e.*, the peptones, peptides, and amino acids, were also found to contribute to the nutrition of fibroblasts.[4] Even when these proteolytic products were derived from a chemically pure protein such as egg albumin crystallized six times, they promoted a considerable growth of fibroblasts from fresh embryonic heart tissue.[5] On the other hand, they caused an exceedingly small proliferation of cells from pure cultures of fibroblasts.[5] When glycocoll and nucleic acid were added to the proteolytic products of crystalline egg albumin or of casein, the resulting mixture was found to promote a considerable growth of pure strains of both normal and sarcomatous fibroblasts.[5] It did not, however, cause a growth at all comparable to that produced by embryo juice. After a time, the cells degenerated, showing that the medium lacked some substance or substances essential for their complete nutrition. These experiments indicated, nevertheless, that it might be possible to synthetize an artificial medium which would prove adequate for the maintenance of cell life and multiplication. Therefore, the effect of adding many biologically important substances to this simple mixture of glycocoll, nucleic acid, and the digestion products of either crystalline egg albumin or casein was studied. Many of them, such as vitamines, iron salts and oxides, cysteine, carbohydrates, cholesterol, etc., had no noticeable beneficial action under the conditions of the experiments. Even though no evidence was obtained of the nutritive value of these substances, the negative results must not be accepted as final, for it is conceivable that any one of them might contribute to the functional requirements of the cells, but that its effect could not be observed in experiments on growth when some other substance necessary for complete nutrition was absent.

It is reasonable to suppose that, in addition to the nitrogenous substances required for the synthesis of protoplasm, certain catalysts or substances should be present in the medium for the regulation of the oxidative or respiratory processes of the cell. It is also probable that a definite oxidation-reduction potential of the medium is as important for cell life as a given hydrogen ion concentration or osmotic

[4] Baker, L. E., and Carrel, A., *J. Exp. Med.*, 1928, xlviii, 533.

[5] Baker, L. E., and Carrel, A., *J. Exp. Med.*, 1928, xlvii, 353.

pressure. For this reason, a study was made of the effect on the growth of fibroblasts of (1) the mineral constituents of liver tissue, (2) of glutathione, and (3) of hemoglobin when added to the incompletely nutritive medium of casein digest, glycocoll, and nucleic acid previously described.[5] The results obtained so far with these substances form the subject of the present communication.

Technique of Cultivating the Tissues.

For this work, a pure strain of sarcomatous fibroblasts from Rat Sarcoma No. 10 of the Crocker Foundation was used. Certain of the experiments were repeated for comparative purposes with a pure strain of normal fibroblasts of the rat. The colonies of fibroblasts were cultivated at 39°C. in flasks 3.5 cm. in diameter, in a coagulum made of 1 cc. of chicken plasma diluted 1:3 with Tyrode solution, and 0.25 cc. of embryo juice. Immediately after coagulation, the serum and embryo juice were removed by washing with Tyrode solution, and 0.5 cc. of the experimental fluid was added. After 24 hours, the coagulum was patched with 0.25 cc. of plasma and 0.25 cc. of embryo juice, and again washed before the addition of the experimental fluid. Every 48 hours, the fluids were removed, the cultures washed in Tyrode solution for 1 hour, and the nutritive media renewed. Every 2 or 3 days, tracings were made under a projectoscope of the growth, the area of which was measured with a planimeter. In each experiment, a colony of cells in active condition was divided into two equal parts. The comparison of the growth-activating action of any two media was made by testing their effect on these two equal parts of the same colony of cells, and each experiment was repeated a number of times. After 8 or 10 days growth, the colonies were removed from the flasks, cut in half, transferred to new flasks, and continued in the same medium, provided the condition of the cells and the area of the growth obtained indicated the desirability of prolonging the experiment.

Action of the Ash of Liver on the Growth of Sarcomatous Fibroblasts.

Although previous experiments with iron salts at many different dilutions had given unsatisfactory results, it still seemed probable, in view of Warburg's[6] experiments, that iron in some form functioned in the respiratory process of the cells. Moreover, it had been found previously that the proteolytic products obtained by peptic or tryptic digestion of liver, which is very rich in iron, sufficed for the complete nutrition of sarcomatous fibroblasts.[7] The recent work of Hart,

[6] Warburg, O., *Biochem. Z.*, 1924, clii, 479; *Pharm. Monats.* 1925, vi, 105.

[7] Baker, L. E., and Carrel, A., *J. Exp. Med.*, 1928, xlvii, 371.

Steenbock, Waddell, and Elvehjem[8] has shown that the ash of liver is effective in curing anemia of rats, and that the copper present in it is as important as the iron. These facts suggested the possibility that both of these elements might play an important rôle in the life of sarcomatous fibroblasts. Experiments were made, therefore, to ascertain whether adding the ash of liver to the mixture of casein digest, glycocoll, and nucleic acid (C. G. N.)[9] increased its nutritive action for sarcomatous fibroblasts.

The ash was prepared by evaporating to dryness 200 cc. of a heated aqueous extract of 120 gm. of liver at pH 5.0, and igniting it in a platinum crucible. When completely oxidized, the ash was dissolved in about 5 cc. of concentrated hydrochloric acid, again evaporated to dryness, and redissolved in sufficient water to make an isotonic solution. Normal sodium hydroxide solution was added to bring the pH approximately to 5.0. If made more alkaline, a precipitate settled out. The solution was, therefore, left acid until after being mixed with the other constituents of the medium, when the whole was adjusted to pH 7.4. Under these conditions, no visible precipitate was formed, except on long standing.

The experimental and control media for cultivating the tissues were made as follows:

Experimental medium	Control medium
1 cc. casein digest[10]	1 cc. casein digest
1.5 cc. glycocoll solution[11]	1.5 cc. glycocoll solution
1.5 cc. nucleic acid solution[12]	1.5 cc. nucleic acid solution
2 cc. ash of liver solution	2 cc. Tyrode solution

The ash caused no significant difference in the area of growth of the tissues (Text-fig. 1). However, the cells in the second passage in the medium containing the ash appeared to be in somewhat better

[8] Hart, E. B., Steenbock, H., Waddell, J., and Elvehjem, C. A., *J. Biol. Chem.*, 1928, lxxvii, 797.

[9] The abbreviation C.G.N. stands in the present paper for a mixture of casein digest, glycocoll, and nucleic acid.

[10] The casein digest was made by digesting at 37°C. 10 gm. of casein with 200 cc. of 0.5 per cent pepsin in N/20 HCl, for 24 hours, boiling, making it isotonic, and adjusting to pH 7.4. It contained 0.7 per cent of nitrogen, 13.5 per cent of which was present as amino nitrogen.

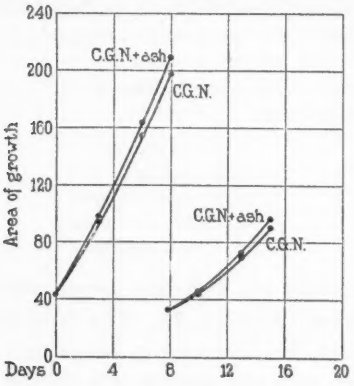
[11] The glycocoll solution contained 50 mg. of glycocoll in 100 cc. of Tyrode solution. It was sterilized by filtering through a Berkefeld filter.

[12] This was a 0.1 per cent solution of thymus nucleic acid in Tyrode solution. It was adjusted to pH 7.4, and sterilized by filtering through a Berkefeld filter.

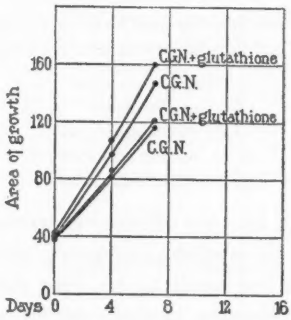
condition than those in the control, and the growth was also slightly thicker. This condition indicated that the mineral constituents of liver were probably beneficial, but if so, other essential substances were still lacking in the medium without which their action could not be definitely demonstrated.

Action of Glutathione on the Growth of Sarcomatous Fibroblasts.

The presence of glutathione in all tissues and its importance in relation to the respiratory processes of the cells have been pointed out



TEXT-FIG. 1.



TEXT-FIG. 2.

TEXT-FIG. 1. Experiments 4612 A and 4639 A. Effect of the ash of liver when added to a mixture of casein digest, glycocoll, and nucleic acid (C.G.N.), on the growth of sarcomatous fibroblasts of the rat.

TEXT-FIG. 2. Experiment 4609 A. Effect of glutathione when added to a mixture of casein digest, glycocoll, and nucleic acid (C.G.N.), on the growth of sarcomatous fibroblasts of the rat.

by Hopkins.[13] What its effect is on the growth of tissues, either through its being used as a food substance, or through its action in regulating the oxidation-reduction potential of the medium, has not been ascertained. Its universal presence in cells indicates either that it must be synthetized by the cell or supplied as such in its nutritive medium. That it probably is an important factor in the multiplica-

[13] Hopkins, F. G., *Biochem. J.*, 1921, xv, 286.

tion of cells is indicated by the fact that it is very abundant in embryonic tissue[14,15] and also in liver,[14] both of which give extracts with marked growth-promoting properties. Therefore, an attempt was made to see if it would increase the growth-promoting power of the mixture of casein digest, glycocoll, and nucleic acid (C. G. N.).

The glutathione in this and the other experiments reported in the present paper was very kindly furnished for this purpose by Dr. Carl Voegtlin of the U. S. Public Health Service at Washington, D. C., and had been shown to have the same composition as the glutathione prepared by Hopkins,[13] namely that of a dipeptide of glutamic acid and cysteine.[16] The solution used contained 20 mg. of glutathione in 10 cc. of Ringer solution and was sterilized by filtering through a Berkefeld filter. Only 10 cc. of solution were prepared at a time and this was kept in a refrigerator until completely used to avoid oxidation changes as far as possible.

The following media were used for the experiment:

Experimental medium	Control medium
1.0 cc. casein digest	1.0 cc. casein digest
1.5 cc. glycocoll solution	1.5 cc. glycocoll solution
1.5 cc. nucleic acid solution	1.5 cc. nucleic acid solution
1.0 cc. glutathione solution	1.0 cc. Tyrode solution

In two out of three experiments, there was a slightly larger growth in the medium containing glutathione (Text-fig. 2). The difference was too small to be very significant, but here again microscopical examination of the colonies showed that the growth was thicker in the medium containing glutathione, and the cells at the edge of the colony were also in somewhat better condition than those in the control medium.

Combined Action of Glutathione and Ash of Liver on the Growth of Sarcomatous Fibroblasts.

Since both the ash of liver and glutathione seemed to increase slightly the nutritive value of the mixture of casein digest, glycocoll, and nucleic acid, the action of the two together was tested.

[14] Thompson, J. W., and Voegtlin, C., *J. Biol. Chem.*, 1926, lxx, 793. Hopkins, F. G., *Biochem. J.*, 1921, xv, 286. Tunnicliffe, H. E., *Biochem. J.*, 1925, xix, 194. Murray, H. A., *J. Gen. Physiol.*, 1926, ix, 621.

[15] It has also been shown by Murray that the glutathione content of embryos varies with age. He suggests that this may be associated with the different rates of growth of embryonic tissue taken from embryos of different ages. Murray, H. A., *J. Gen. Physiol.*, 1926, ix, 621.

[16] Johnson, J. M., and Voegtlin, C., *J. Biol. Chem.*, 1927, lxxv, 703.

The media were made as follows:

Experimental medium	Control medium
1.0 cc. casein digest	1.0 cc. casein digest
1.5 cc. glycocoll solution	1.5 cc. glycocoll solution
1.5 cc. nucleic acid solution	1.5 cc. nucleic acid solution
1.5 cc. glutathione solution	3.0 cc. Tyrode solution
1.5 cc. ash of liver solution	

In this case, there was a very noticeable difference in the nutritive value of the two media. At the end of the first 7 days, there was a slightly larger growth in the colonies cultivated in the medium contain-

TABLE I.

Effect of Glutathione and Ash of Liver When Both Are Added to a Mixture of Casein Digest, Glycocoll, and Nucleic Acid, on the Growth of Sarcomatous Fibroblasts.

Experiment No.	Time of growth	Area of growth* in C. G. N. Control	Area of growth* in C. G. N., ash, and glutathione. Experiment	Ratio: $\frac{E}{C}$	Remarks
	days				
4617 A	7	125	150	1.2	First passage
4618 A	7	110	122	1.1	" "
4637 A-1	7	90	98	1.1	Second " ; thicker growth in experiment
4637 A-2	7	91	113	1.2	Second passage; thicker growth in experiment
4638 A-1	9	80	100	1.25	Second passage; thicker growth in experiment
4638 A-2	9	102	143	1.4	Second passage; thicker growth in experiment

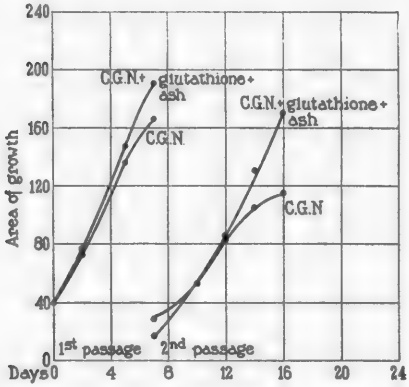
* This is the area in square centimeters of the image of the new growth as drawn with a projectoscope, which magnifies the size approximately 250 times.

ing glutathione and ash of liver than in the controls. The new cells around the edge of the colony were in better condition than those of the controls in the C.G.N. mixture,[9] although there were a number of dead cells at the center of the colonies. The center was then removed, and the colonies were transferred to new flasks to continue the experiment. At the end of 16 days, there was a very decided difference in their appearance. Those in the medium containing the glutathione and ash were not only larger in area (Text-fig. 3, Table I), but con-

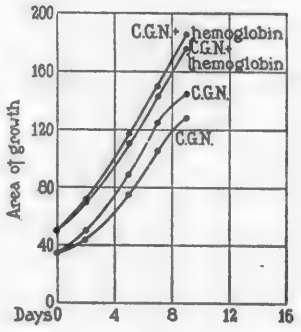
tained a much thicker growth and the cells were in far better condition than those of the colonies of the controls. Their condition was not as good, however, as those of the same strain which were cultivated in embryo juice.

Action of Hemoglobin on the Growth of Sarcomatous Fibroblasts.

The hemoglobin for the experiments was obtained from the red cells of heparinized rat blood.



TEXT-FIG. 3.



TEXT-FIG. 4.

TEXT-FIG. 3. Experiments 4617 A and 4638 A. Effect of ash of liver and glutathione when added to a mixture of casein digest, glycocoll, and nucleic acid (C.G.N.), on the growth of sarcomatous fibroblasts of the rat. The growth in the C.G.N., glutathione, and ash was much thicker than that in C.G.N. alone.

TEXT-FIG. 4. Experiment 4685 A. Effect of rat hemoglobin when added to a mixture of casein digest, glycocoll, and nucleic acid (C.G.N.), on the growth of sarcomatous fibroblasts of the rat.

The cells were separated from the plasma by centrifugation, washed three times in Ringer solution, and centrifuged. 1.5 cc. from the lower layer of cells was removed by a capillary pipette and hemolyzed by the addition of 9 cc. of water, and again centrifuged. 1 cc. of the clear supernatant fluid was diluted with 9 cc. of Ringer solution. The final preparation contained 0.029 per cent of nitrogen.

When this hemoglobin was added to the mixture of casein digest, glycocoll, and nucleic acid, a very small but decided increase in the area of growth was obtained (Text-fig. 4, Table II). In one experi-

ment, after five passages a small cell proliferation was still taking place in the medium containing the hemoglobin, although the colonies in the control had died. Here also it seemed probable that hemoglobin had some beneficial effect, but that the full extent of its action was not demonstrated because some other essential substance was lacking.

Combined Action of Ash of Liver, Glutathione, and Hemoglobin on the Growth of Sarcomatous Fibroblasts.

Since the preceding experiments indicated that each of these substances had a slightly beneficial effect on the growth of fibroblasts,

TABLE II.

Effect of Hemoglobin When Added to Casein Digest, Glycocoll, and Nucleic Acid, on the Growth of Sarcomatous Fibroblasts.

Experiment No.	Time of growth	Area of growth* in C. G. N. Control.	Area of growth* in C. G. N. and hemoglobin. Experiment	Ratio: $\frac{E}{C}$	Remarks
	days				
4685 A-1	10	91	126	1.4	First passage
4685 A-2	10	107	140	1.3	" "
4697 A-1	10	38	57	1.5	" "
4697 A-2	10	26	28	1.1	" "
107 J	7	94	139	1.5	" "
131 J	9	69	96	1.4	Second "
155 J	6	30	65	2.2	Third "
178 J	9	15	75	5.0	Fourth "
208 J	6	0	14	∞	Fifth "

their combined action was tested by adding all three of them to the mixture of casein digest, glycocoll, and nucleic acid, and comparing the growth-promoting power of this mixture[17] with that of casein digest, glycocoll, and nucleic acid alone.

The media used were made as follows:

Experimental medium	Control medium
1.0 cc. casein digest	1.0 cc. casein digest
1.5 cc. glycocoll solution	1.5 cc. glycocoll solution
1.5 cc. nucleic acid solution	1.5 cc. nucleic acid solution
1.0 cc. ash of liver solution	3.0 cc. Tyrode solution
1.5 cc. glutathione solution	
0.5 cc. hemoglobin solution	

[17] This mixture will be designated by the abbreviation C.G.N.A.G.H.

Very striking results were obtained. In the first passage, the rate of growth of the tissues in the mixture containing hemoglobin, ash, and glutathione greatly exceeded that in the C.G.N. mixture (Text-fig. 5). In some cases, the difference was as great as 90 and 100 per cent (Table III). In the second and third passages, still greater differences were observed. The rate of growth in the mixture of casein digest,

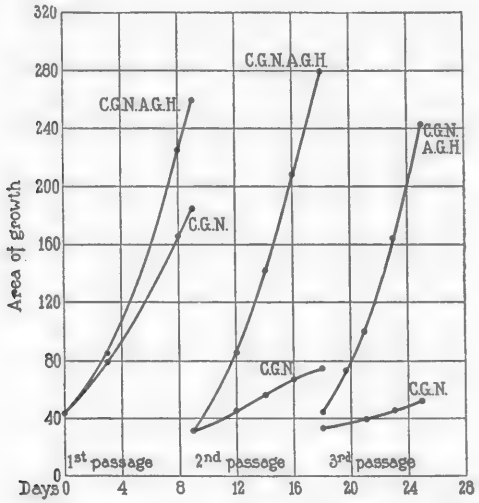
TABLE III.

Effect of Hemoglobin, Glutathione, and Ash of Liver When Added to a Mixture of Casein Digest, Glycocoll, and Nucleic Acid, on the Growth of Sarcomatous Fibroblasts.

Experiment No.	Time of growth	Area of growth* in C. G. N. Control	Area of growth* in C. G. N. A. G. H. Experiment	Ratio: $\frac{E}{C}$	Remarks
	days				
4621 A-1	9	138	255	1.8	First passage; cells better in experiment
4621 A-2	9	126	213	1.7	" "
4654 A-1	9	69	138	2.0	" "
4654 A-2	9	70	117	1.7	" "
4651 A-1	9	36	195	5.4	Second passage; cells better in experiment
4651 A-2	9	34	191	5.6	" "
4651 A-3	9	49	246	5.0	" "
4651 A-4	9	35	219	6.2	" "
4676 A	7	19	190	10.0	Third passage; cells better in experiment
4679 A	7	57	133	2.3	Second passage; cells better in experiment
4680 A	7	35	160	4.6	Third passage; cells better in experiment

glycocoll, and nucleic acid decreased progressively as the time increased, and the cells gradually degenerated. In the mixture containing the ash, glutathione, and hemoglobin, the initial rapid rate of growth continued in the second and third passages, giving large colonies with thick growth and very active cells in good condition (Text-fig. 5). In fact, the growth appeared to be fully as great as that which usually takes place in embryo juice. Therefore, after the second passage, some of the colonies which had been growing for 18

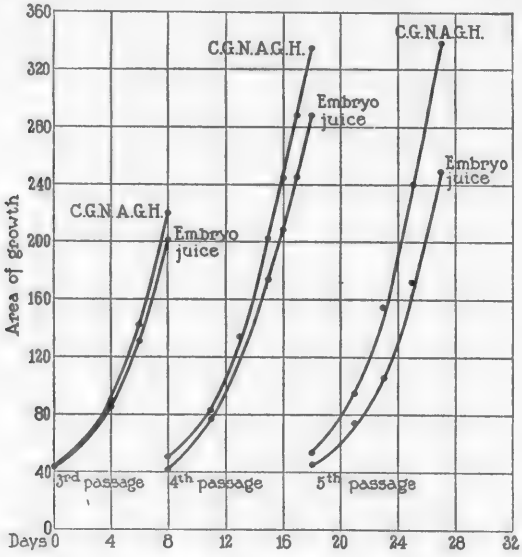
days in this mixture (C.G.N.A.G.H.) were divided. One half of each was cultivated in the same medium while the other half was cultivated as a control in embryo juice. The area of growth in the third, fourth, and fifth passages, or for 27 days more, proved to be fully as great in this artificial medium as in embryo juice (Text-fig. 6, Table IV). The nutritive value of this artificial medium is, however, not as great as that of embryo juice. The colonies in the latter were composed of a fine network of cells with apparently clear cytoplasm,



TEXT-FIG. 5. Experiments 4621 A, 4651 A, and 4676 A. Effect of ash of liver, glutathione, and rat hemoglobin (A.G.H.) when added to a mixture of casein digest, glycocoll, and nucleic acid (C.G.N.), on the growth of sarcomatous fibroblasts of the rat.

all in healthy condition. Those in the C.G.N.A.G.H. mixture, although larger than those in embryo juice, were composed of cells that showed from the beginning many tiny fat droplets and numerous cytoplasmic granules. This seems to be a characteristic appearance of cells cultivated in proteolytic products and is not necessarily a condition of degeneration, but is often associated with rapid proliferation. For the first four passages, the cells were small, oval, grew compactly, and were very similar to the sarcomatous fibroblasts

cultivated in tryptic digests of liver. After the fourth passage, the growth was much thinner, and a considerable number of dead cells were scattered throughout the culture. In any passage after 8 days of growth in one flask, the central or original fragment of the colony was surrounded with dead cells. The cells further out, however, appeared to be in a healthy condition and continued in an active state



TEXT-FIG. 6. Experiments 4671 A and 4690 A. Comparison of the growth of sarcomatous fibroblasts of the rat in a mixture of casein digest, glycocoll, nucleic acid, ash of liver, glutathione, and rat hemoglobin (C.G.N.A.G.H.) during the third, fourth, and fifth passages in this mixture, with that in embryo juice.

of proliferation. The presence of dead cells within these colonies seems to be associated with the presence of glutathione, as this condition prevailed in the other glutathione experiments in which hemoglobin and ash were not used. When transplanted to a new flask in a new plasma coagulum, a rapid growth of the colonies occurred in which no dead cells appeared for many days. The exact cause of this phenomenon has not been ascertained. It is evident that this artificial medium either still lacks some substance essential for the

proper functioning of the cells or else contains some toxic substances. It is also highly probable that the proportion of the various constit-

TABLE IV.

Comparison of the Growth of Sarcomatous Fibroblasts in a Mixture of Casein Digest, Glycocoll, Nucleic Acid, Ash of Liver, Glutathione, and Hemoglobin, with That in Embryo Juice.

Experiment No.	Time of growth	Area of growth* in embryo juice	Area of growth* in C. G. N. A. G. H. Experiment	Ratio: $\frac{E}{C}$	Remarks
	days				
4679 A	7	98	133	1.4	Second passage in C. G. N. A. G. H.
4671 A	8	160	158	0.99	Third " " "
4690 A	10	248	277	1.1	Fourth " " "
4690 A	10	186	278	1.5	" " " "
4711 A	9	161	283	1.8	Fifth " " "
4711 A	9	205	307	1.5	" " " "
4712 A	7	146	186	1.3	" " " "

TABLE V.

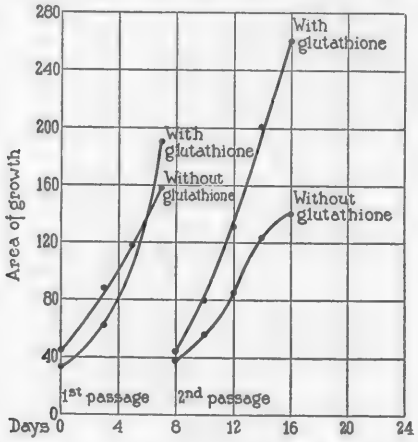
Comparison of the Growth of Sarcomatous Fibroblasts in a Mixture of Casein Digest, Glycocoll, Nucleic Acid, Ash of Liver, and Hemoblogin, with and without the Addition of Glutathione.

Experiment No.	Time of growth	Area of growth* without glutathione. Control	Area of growth* with glutathione. Experiment	Ratio: $\frac{E}{C}$	Remarks
	days				
4672 A	8	96	223	2.3	First passage without glutathione; cells better in experiment
4680 A	7	113	160	1.4	First passage without glutathione
4693 A-1	7	100	174	1.7	Second " " " cells poor in control
4693 A-2	7	92	214	2.3	Second passage without glutathione; cells poor in control
4702 A	9	64	169	2.6	Second passage without glutathione

uents to each other is not the optimum for maintaining the cells in the best condition.

The Rôle Played by the Ash and the Glutathione in the Artificial Medium.

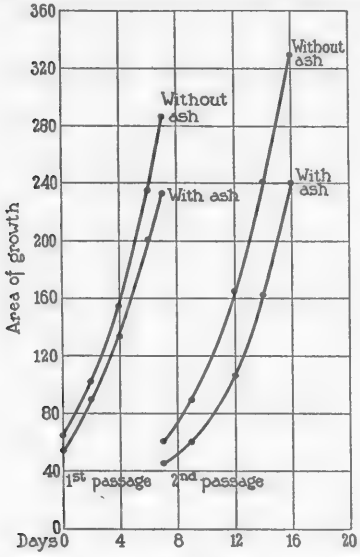
Although the combined action of the ash of liver, glutathione, and hemoglobin, when added to casein digest, glycocoll, and nucleic acid, caused a rapid proliferation of fibroblasts, it does not necessarily follow that all three of these contributed to the result. The above experiments left no doubt regarding the beneficial action of hemoglobin. In order to ascertain conclusively whether both the ash and glutathione were utilized, this artificial medium was tested for its growth-promoting power on sarcomatous fibroblasts, with and without the



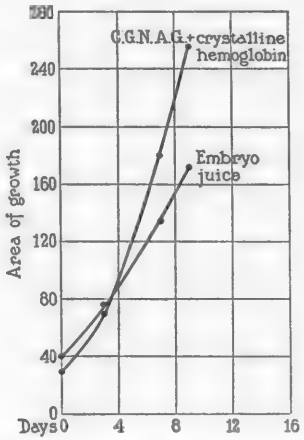
TEXT-FIG. 7. Experiments 4680 A and 4693 A. Comparison of the growth of sarcomatous fibroblasts of the rat in a mixture of casein digest, glycocoll, nucleic acid, ash of liver, and rat hemoglobin, with and without the addition of glutathione.

glutathione, and also with and without the ash. The omission of the glutathione from the medium resulted in a marked decrease of growth, especially in the second passage (Text-fig. 7, Table V). A rapid degeneration of the cells also took place. On the other hand, the colonies cultivated without the ash grew fully as large as those with it, and their cells seemed to have fewer cytoplasmic granules (Text-fig. 8, Table VI). Since the ash had caused a slight increase in the nutritive value of the C.G.N. mixture, and better results were obtained with glutathione and ash than with only the glutathione, it must be con-

cluded that some mineral constituent of the ash, probably the iron, does play some part in the functioning of the cells, but that the hemoglobin furnishes the necessary iron in a more utilizable form. There is also the possibility that the quantity of mineral constituents



TEXT-FIG. 8.



TEXT-FIG. 9.

TEXT-FIG. 8. Experiments 4694 A and 4709 A. Comparison of the growth of sarcomatous fibroblasts of the rat in a mixture of casein digest, glycocoll, nucleic acid, glutathione, and rat hemoglobin, with and without the addition of ash of liver.

TEXT-FIG. 9. Experiment 4607 A. Comparison of the growth of sarcomatous fibroblasts of the rat in embryo juice and in a mixture of casein digest, glycocoll, nucleic acid, ash of liver, glutathione, and hemoglobin recrystallized three times.

of the ash required by the cells is so small that they have absorbed from their previous medium a sufficient supply to function for a considerable period.

The Effect of Using Crystalline Hemoglobin in the Artificial Medium.

The same rapid proliferation of cells took place when a solution of rat hemoglobin crystallized three times was substituted for the

original solution of hemoglobin (Text-fig. 9). Therefore, it is the hemoglobin itself, and not any other substance derived from the hemolyzed red cells, that brings about this action. However, the cells in these experiments were not in quite as good condition as in the previous ones. Whether the crystallization removed some substance which accounts for this has not been ascertained as yet.

Action of Glutathione, Ash of Liver, and Hemoglobin on the Proliferation of Normal Fibroblasts.

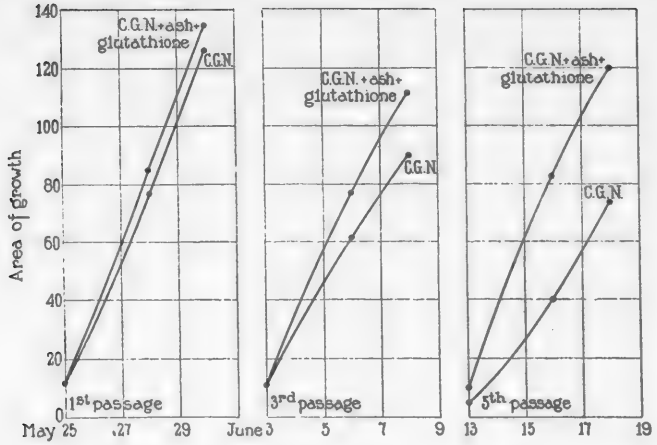
In some cases, experiments parallel with the above were carried out simultaneously on normal fibroblasts of the rat. For these cells

TABLE VI.

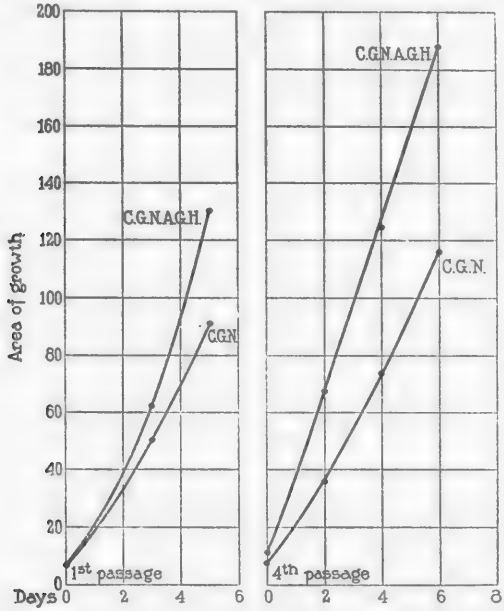
Comparison of the Growth of Sarcomatous Fibroblasts in a Mixture of Casein Digest, Glycocoll, Nucleic Acid, Glutathione, and Hemoglobin, with and without the Addition of Ash of Liver.

Experiment No.	Time of growth	Area of growth* without ash. Control.	Area of growth* with ash. Experiment	Ratio: $\frac{E}{C}$	Remarks
	days				
4677 A	7	116	121	1.0	First passage without ash
4694 A	7	220	178	0.81	Second " " "
4701 A	9	221	205	0.93	First " " "
4709 A-1	9	270	145	0.54	Second " " "
4709 A-2	9	194	195	1.0	" " " "
4713 A-1	8	138	186	1.3	" " " "
4713 A-2	8	149	188	1.3	" " " "

also, the addition of ash of liver, glutathione, and hemoglobin to the mixture of casein digest, glycocoll, and nucleic acid increased the nutritive value of the mixture (Text-figs. 10 and 11). This artificial medium is not as well adapted to the cultivation of the normal as of the sarcomatous fibroblasts. The cells in the experiments and also those in the controls filled with fat, and the colonies presented a much more opaque appearance than those cultivated in embryo juice. This difference between normal and sarcomatous fibroblasts has been observed in all cases where attempts have been made to cultivate them in media containing hydrolyzed proteins, as for instance in the peptic and tryptic digests of liver.[4,7] Nevertheless, the normal fibroblasts proliferated for several passages in this medium.



TEXT-FIG. 10. Experiments 4615 A, 4646 A, and 4666 A. Effect of ash of liver and glutathione when added to a mixture of casein digest, glycocoll, and nucleic acid (C.G.N.), on the growth of *normal* fibroblasts of the rat.



TEXT-FIG. 11. Experiments 4659 A and 4665 A. Effect of ash of liver, glutathione, and rat hemoglobin when added to a mixture of casein digest, glycocoll, and nucleic acid, on the growth of *normal* fibroblasts of the rat.

DISCUSSION.

The hypothesis was suggested above that a definite oxidation-reduction potential in the medium might be as important for the continuous proliferation of fibroblasts as a given hydrogen ion concentration or osmotic pressure. It is quite possible that the glutathione and hemoglobin act not only by regulating the respiration and oxidation-reduction reactions within the cell, but also by creating a desirable oxidation-reduction potential of the medium. They also furnish necessary materials for the synthesis of protoplasm. A study is being made of the oxidation-reduction potential of this medium and of the others in which cell proliferation takes place. It does not necessarily follow from these experiments that hemoglobin as such is essential for the proliferation of sarcomatous fibroblasts. It has already been shown that they proliferate indefinitely in the tryptic and peptic digests[4,7] of liver in which the protein part of the molecule has been destroyed by enzymatic action. Experiments will be made to ascertain whether an iron pyrrol compound such as hemin can replace the hemoglobin in this medium. The growth-promoting action of glutathione is interesting in connection with the data recently published on the nitrogen metabolism of fibroblasts, in which it was shown that a certain amount of peptide nitrogen was required for their continuous proliferation.[4] The growth-promoting action of liver digests was destroyed by complete hydrolysis with hydrochloric acid.[4] It is quite probable that the deleterious action of acid hydrolysis was due in part at least to its destruction of glutathione, and that this substance cannot be resynthetized by these cells. It has been suggested by Voegtlin and Thompson,[18] as a result of their work on the glutathione content of tissues of tumor-bearing animals, that the rapid growth of tumor, especially in sarcomatous rats, deprives other tissues of their normal content of glutathione and brings about a state of malnutrition. They could not ascertain whether this effect was due to the utilization of glutathione and other nutrients by the growing tumor or to the toxic action of necrotic material. These experiments prove that glutathione is required as a nutrient substance by sarcoma cells. There is, however, no evidence as yet as to whether

[18] Voegtlin, C., and Thompson, J. W., *J. Biol. Chem.*, 1926, lxx, 801.

the requirement of sarcomatous fibroblasts exceeds that of normal fibroblasts. Even though their requirement should be identical, it is probable that rapid multiplication of tumor tissue could produce this effect.

The experiments cited above are interesting in connection with the question of respiration or oxidation within the cell. The continuous life and multiplication of cells *in vitro* would most certainly indicate that a respiration normal for the tissue in question, and oxidations essential to life processes were occurring. For the life of fibroblasts *in vitro*, both glutathione and an iron compound are necessary. Iron in the form of hemoglobin, or hemoglobin hydrolyzed by enzymes, suffices, while iron in the inorganic form has failed to do so in all experiments tried thus far. These experiments, therefore, are in accord with the observations of Meyerhof,[19] and of Hopkins [20] on the importance of compounds containing the SH groups to cell oxidations, and also with those of Warburg[21] on the importance of iron or an iron-containing compound as a respiratory ferment.

SUMMARY.

1. It has been shown that the ash of liver, hemoglobin, and glutathione each exerts a very slight beneficial effect on the growth of sarcomatous fibroblasts of the rat, or on the condition of their cells when cultivated in a synthetic medium.

2. The addition of all three of these substances, or of only glutathione and hemoglobin, to a mixture of casein digest, glycocoll, and nucleic acid gives a medium in which sarcomatous fibroblasts of the rat proliferate for a considerable time as rapidly as in embryo juice.

3. The mixture is not as adequate a nutritive medium as embryo juice, for after a time dead cells are found surrounding the central fragment of the culture, and after several passages the growth becomes thinner.

4. The hypothesis is suggested that glutathione and hemoglobin may function not only by regulating the respiration and oxidation-

[19] Meyerhof, O., *Arch. ges. Physiol.*, 1923, cxcix, 531.

[20] Hopkins, F. G., *Biochem. J.*, 1925, xix, 787.

[21] Warburg, O., *Biochem. Z.*, 1924, clii, 479; *Naturwissensch.*, 1928, xvi, 345.

reduction reactions within the cell, but also by regulating the oxidation-reduction potential of the medium.

5. It is suggested that the failure to obtain growth of fibroblasts in mixtures of amino acids or of the products of complete acid hydrolysis of proteins is in part due to the absence of glutathione, and that this substance is not synthetized by fibroblasts.

6. The growth of normal fibroblasts of the rat is also increased by the addition of the above mentioned substances to a synthetic medium.

STUDIES ON IMMUNOLOGICAL RELATIONSHIPS AMONG THE PNEUMOCOCCI.

III. Relationship between a Variety of Saccharomyces cerevisiæ and the Type II Variety of Diplococcus pneumoniæ (Pneumococcus).

By JOHN Y. SUGG and JAMES M. NEILL, Ph.D.

(*From the Department of Bacteriology and Immunology, Vanderbilt University Medical School, Nashville.*)

(Received for publication, October 18, 1928.)

INTRODUCTION.

The present paper reports evidence of an immunological relationship between one variety or type of the yeast *Saccharomyces cerevisiæ* and the Type II variety of *Diplococcus pneumoniæ* (Pneumococcus). The immunological interrelationships among the different yeasts have never been well established, but it is certain that all of the yeasts included by the species term *Saccharomyces cerevisiæ* are not immunologically identical. Only one strain or variety was used in most of our investigation. Experiments were made with a number of the recognized varieties of the species, that differ from each other in fermentation and other cultural properties, but the present data are not sufficient to furnish definite proof of the possession or lack of serological relationship of these other yeast varieties to Type II pneumococci. It is necessary, therefore, to limit the present paper to the relationship exhibited by one variety of *Saccharomyces cerevisiæ* that was derived from commercial (Fleischmann's) dried yeast.

The immunological relationship between the yeast and the Type II pneumococcus may be reported by the presentation of two kinds of data: (1) the reactions of the yeast with Type II antipneumococcus serum; (2) the reactions of Type II pneumococci with the antiyeast serum. In the following report the reactions of the yeast with the antipneumococcus serum are presented first, although the evidence

obtained from that source is less convincing than that obtained from the reactions of the pneumococci with the antiyeast serum.

EXPERIMENTAL.

Methods.—All tests for agglutination of the yeast were made with glucose meat extract broth cultures of a yeast strain derived from a single cell culture isolated from Fleischmann's dried yeast by Dr. Roy C. Avery (1). The antiyeast immune sera were produced by immunization of rabbits with two sorts of material: (1) washed yeast cells from glucose agar cultures of the pure single cell strain; (2) the dried yeast cells contained in the commercial preparation. The yeast suspensions were of approximately equal turbidity and were heated for 5 to 10 minutes at 100°C. The immunization consisted of one to four courses of 6 daily intravenous injections with a rest period of 7 to 10 days between courses; with most rabbits test bleedings were made at the end of the rest period following each course of injections.

The immunization with the dried yeast produced the more potent antiserum and in fact, antipneumococcus agglutinins were obtained only from rabbits immunized with the dried material. A possible objection that might be raised to basing the antipneumococcus relationship upon these antiyeast sera is answered by the fact that the antisera produced by immunization with the dried yeast can be exhausted of antibodies reactive with either pneumococci or with yeast by absorption with yeast cells derived from the pure single cell culture alone.

The production of the antiyeast sera was complicated by the death of many of the rabbits during the immunization. The yeast suspensions did not seem to be primarily toxic for no rabbits were killed by the first course of injections. During the second and third courses (after rest periods of 7 to 10 days) a number of rabbits were killed with anaphylactoid symptoms upon injection of the yeast. While the mechanism of the reaction was not studied intensively, it was the second or third rather than the first injection following the rest period which caused the death of most of the rabbits. It is possible that the death of the rabbits was due to a true anaphylactic reaction that occurred from the second or third injection when the circulating antibodies had been removed by the first injection following the rest period. The anaphylactic or anaphylactoid deaths were more frequent with animals injected with the yeast cells from the agar cultures than with animals injected with the dried yeast.

A means of decreasing the likelihood of anaphylactic or anaphylactoid death of rabbits during immunization was observed during the latter part of the investigation. Since the death of animals utilized for production of antisera is a serious complication in any investigation, it seems desirable to report the procedure as a possible means of preventing the acute death of rabbits during the later stages of their immunization with other kinds of antigens. At the suggestion of Dr. Walter E. Garrey attempts were made to save the life of the rabbits by intravenous in-

jection of glucose solution. A number of times, rabbits which were exhibiting marked symptoms and which indeed were apparently about to die, recovered rapidly when approximately 5 cc. of 10 per cent glucose was injected intravenously. Intraperitoneal injection of the same amount of glucose was also given to most of these animals immediately or soon after the intravenous injection.

Agglutination of the Yeast by Type II Antipneumococcus Serum.

1. Agglutination by Type II Antipneumococcus Immune Horse Serum.—The tests were made with 3 different Type II antipneumococcus, 7 anti-Type I, 1 anti-Type III, and 4 antimeningococcus immune horse sera. When observed after 2 hours at 37°C., the yeast was agglutinated only by anti-Type II serum. The agglutination occurred with undiluted and one-fifth dilution but not with one-tenth dilution of serum. None of the other horse sera showed any agglutination. The distinction became somewhat less clean-cut after ice box storage which resulted in slight agglutination of the yeast cells in four of the eleven control horse sera; this occurred only in undiluted serum and was never comparable to that in any of the three anti-Type II immune horse sera. These results with the antipneumococcus serum from horses appeared to furnish definite evidence of a serological relationship between the Type II pneumococcus and the yeast.

2. Agglutination by Immune and by Normal Rabbit Serum.—In the experiments in which the relationship between the yeast and the Type II pneumococci was studied by tests of yeast agglutination with Type II antipneumococcus rabbit serum, it was found that the serum of many normal rabbits possessed the property of agglutinating the yeast. While these experiments contributed no proof of relationship between the yeast and the Pneumococcus, the results summarized in Tables I and II are important as experimental facts concerning the irregularities in the yeast-agglutinating capacities of serum from different individual rabbits.

Table I presents the results of agglutination tests made with several bleedings from 168 different rabbits. It is evident that the yeast was definitely agglutinated by the serum of some, but not by the serum of other rabbits. Due to the number of animals tested, the most satisfactory method of presenting the results is in the form of the percentage of animals whose serum agglutinated the yeast in the different test dilutions. In tests with undiluted, one-fifth, and one-tenth dilutions of serum, there was no significant difference in the percentage of yeast

agglutinators between the group of 33 rabbits immunized with Type II pneumococci, the group of 75 immunized with other bacteria, or the group of 60 rabbits that had not been immunized at all. The fact that serum from a few of the rabbits immunized with pneumococci agglutinated the yeast in higher dilutions than did serum from any of the other rabbits must be considered of little significance in the absence of tests with serum obtained from bleedings of the same animals before their immunization. While impossible to test normal bleedings of these rabbits, there were available two or three bleedings taken during different stages of the antipneumococcus immunization of almost all the pneumococcus group of rabbits. Repeated tests of the early and late bleedings of these rabbits showed

TABLE I.

Comparison of Yeast-Agglutinating Property of Serum from "Normal" and Immunized Rabbits.

Rabbits		Percentage of animals whose serum agglutinated yeast in different dilutions				
Number in group	Previous immunization	Dilution of serum				
		Undil.	1/5	1/10	1/20	1/40
		*per cent**	*per cent*	*per cent*	*per cent*	*per cent*
33	Type II pneumococci	42	30	24	15	9
39	Type I and III pneumococci	40	36	28	15	3
36	Diphtheria, anthrax, or tetanus bacilli	36	22	17	8	0
60	"Normal" or non-immunized	43	35	22	8	0
168	Total immune and normal	40	32	22	11	3

* Per cent of individuals in respective group whose serum agglutinated yeast cells; *i.e.* $\left(\frac{\text{Number of yeast agglutinators}}{\text{Number of rabbits in group}}\right)$.

no significant differences in the yeast-agglutinating titre of the different samples. Since the antipneumococcus agglutinins did increase between the early and late periods of immunization of the same animals, it seems probable that the injection of the rabbits with Type II pneumococci had little if anything to do with the titre of yeast agglutinins.

Table II consists of a summary of tests of the persistence of the yeast-agglutinating property of the serum of individual "normal" rabbits. Serum was obtained from 24 non-immunized rabbits by two bleedings separated by an interval of 6 weeks, and the two samples from the same rabbits tested against the yeast culture. All the rabbits whose serum possessed yeast agglutinins at the time of the first bleeding, likewise possessed them 6 weeks later; and none of the rabbits lacking

yeast agglutinins at the time of one of the bleedings possessed them at the time of the other bleeding. Moreover, the actual titre of the yeast agglutination was surprisingly constant for each individual rabbit, a significant difference in the titres of the two bleedings from the same animal being found with only 4 of the 24 rabbits of the series. This uniformity in the lack or possession of the yeast-agglutinating capacity over a period of 6 weeks is of some significance as proof that the agglutinating property is a characteristic of the individual rabbit, that it is not gained or lost in short periods of time. The so called "normal agglutinins" which are frequently encountered in tests against many kinds of bacteria are always difficult to explain. In the present instance, there is no knowledge of either the nature or of the origin of the property responsible for agglutination of the yeast. It is possible that the agglutination by serum from one "normal" rabbit and not by that from another rabbit, is due to some non-specific constituent

TABLE II.

Relative Constancy of the Yeast-Agglutinating Property of the Serum of Individual "Normal" Rabbits from 2 Bleedings Separated by an Interval of 6 Weeks.

Animals showing no change					Animals showing slight change			
Titre of both samples of serum				Total number of animals	Change in titre of serum between first and second bleedings			Total number of animals
No agglutination	Serum dilution				Serum dilution			
	1/5	1/10	1/20		1/5 to 1/20	1/10 to 1/5	1/20 to 1/10	
14	3	1	2	20	1	1	2	4

or physical property that varies in different individual rabbits. It is not impossible, however, that the yeast agglutination may be due to specific antibodies arising from some unrecognized chronic infection of individual rabbits with microorganisms possessing a serological relationship to the yeast.

Agglutination and Protection Tests with Type II Pneumococci against Antiyeast Serum.

The tests of the yeast cells against Type II antipneumococcus serum from horses had furnished some evidence of serological relationship between the yeast and the Type II pneumococci. More convincing evidence of the relationship was furnished by the reactions of the Type II bacteria when tested against the antiyeast serum. The degree of reactivity of the antisera from two of the rabbits immunized

with yeast was comparable to that of the antisera often obtained by immunization of rabbits with Type II pneumococci themselves; broth cultures of Type II pneumococci being agglutinated by 1/40 dilution of the antiserum and mice being protected against 1×10^{-3} cc. of broth cultures of the Type II bacteria. There was considerable variation in the antipneumococcus value of the serum obtained from different rabbits immunized with the yeast. Some did not agglutinate broth cultures of the pneumococci, but the serum of all seven rabbits which survived immunization with dried yeast did contain

TABLE III.

Absorption of Antiyeast Immune Serum with Yeast Cells and with Types I, II, and III Pneumococci.

Antiyeast serum absorbed with	Yeast agglutination		Type II pneumococcus agglutination	
	Serum dilution		Serum dilution	
	1/5	1/320	2/5	1/20
Yeast cells	0	0	0	0
Type II pneumococci	+++	+	0	0
Type I pneumococci	+++	+	+++	+
Type III pneumococci	+++	+	+++	+
Unabsorbed	+++	+	+++	+

some antibodies reactive[4,9] with Type II pneumococci as indicated by their specific passive protection of mice against at least 1×10^{-4} or 1×10^{-5} cc. of virulent Type II pneumococci.

Pneumococcus Type Specificity of the Antiyeast Immune Sera.

While the antiyeast sera contained antibodies reactive with Type II pneumococci, they were apparently devoid of antibodies reactive with either Type I or Type III. The same antisera that agglutinated and protected against different strains of the Type II variety, always proved entirely non-reactive with the other types of pneumococci. This is an important index that the relationship of the yeast to the Type II pneumococcus is an S-anti-S relationship which does not extend to other serological varieties of pneumococci.

Absorption of Antiyeast Immune Sera.

Antiyeast immune (rabbit) serum was absorbed with suspensions of heated yeast cells and with suspensions of heated Type II pneumococci. For controls on the pneumococcus type specificity, the same antiyeast sera were absorbed with suspensions of Type I and of Type III pneumococci. The results of the several experiments made were the same. As shown in Table III, absorption of the antiyeast serum with the homologous yeast cells completely exhausted it, not only of agglutinins for the yeast but also of agglutinins for Type II pneumococci. On the other hand, repeated absorption with the Type II pneumococcus cells removed only the anti-Type II agglutinins and had little, if any, effect upon the yeast-agglutinating capacity of the serum. In contrast to the exhaustion of the Type II agglutinins by absorption with the Type II bacteria, absorption with Types I and III pneumococci did not affect the Type II-agglutinating capacity of the antiyeast immune sera.

Absorption of Type II Antipneumococcus Immune Horse Serum.

Reciprocal absorption experiments were made with Type II antipneumococcus immune horse serum by absorbing the Type II antiserum with suspensions of yeast and with suspensions of Types I, II, and III pneumococci. The results were analogous to those obtained in the absorption of the antiyeast serum; *i.e.*, absorption of the anti-Type II serum with the Type II bacteria exhausted the capacity of the serum to agglutinate either Type II pneumococci or the yeast; absorption with the yeast cells removed the agglutinins for the yeast but had little, if any, effect upon the Type II-agglutinating capacity; absorption with Types I and III pneumococci had no significant influence upon the agglutination of either the yeast or the Type II bacteria. The exhaustion of the anti-Type II serum by absorption with the homologous Type II pneumococci was to be expected, but the removal of the yeast agglutinins from the antipneumococcus serum by homologous (Type II) absorption is of interest as an index that the yeast-agglutinating capacity of the anti-Type II immune horse serum is due to antibodies reactive with the Type II bacteria that were utilized in the immunization by which the antipneumococcus serum was prepared.

The results of reciprocal absorption of both the antiyeast and the antipneumococcus immune sera are the same as those usually obtained in reciprocal absorption experiments with immunologically related, but different, kinds of bacteria. The similarity in the results is an interesting example of the fact that the same immunological relationships demonstrable between bacteria are likewise demonstrable between antigens derived from microorganisms as biologically unrelated as are the yeasts and the bacteria.

DISCUSSION.

The preceding experiments dealt with the immunological properties of one variety or type of the *Saccharomyces cerevisiæ* species of yeast. Evidence of immunological relationship of this variety of yeast to the Type II variety of Pneumococcus was furnished by the reactions of the yeast and of the pneumococci in the homologous and heterologous (antiyeast and antibacterial) immune sera.

The data obtainable from the reactions in the antipneumococcus serum were necessarily limited to the immune serum from horses, for the yeast-agglutinating capacity of the serum from many normal rabbits complicated the interpretation of the experiments with antipneumococcus serum from immune rabbits. However, anti-Type II serum from immune horses gave clean-cut results; although definite only in one-fifth dilution of the serum, the yeast agglutination occurred in all of the Type II horse sera tested and not under the same conditions in the serum from eleven other horses immunized with other bacteria. The evidence obtainable from the reactions of Type II pneumococci with potent antiyeast sera, is convincing, for the serum of some rabbits immunized with dried yeast cells approached the anti-Type II potency of the serum obtained by immunization with the Type II bacteria themselves. It is interesting to observe that there was no regular relation between the anti-Type II pneumococcus and the antiyeast potencies of the individual immune sera, *i.e.*, the antiyeast sera most reactive with the yeast were not always the ones most reactive with the Type II pneumococci. The irregularity in the relation potencies (ratio of $\frac{\text{Anti-Type II bacteria potency}}{\text{Antiyeast potency}}$) of the antiserum from individual rabbits immunized with yeast probably

represents differences in the individual responses of rabbits to different antigenic constituents of the yeast.

There is every reason to believe that the relationship of the yeast to the Type II pneumococcus in an S-anti-S relationship. Pure solutions of the S substance from yeast or from the Type II bacteria were not available. However, a number of tests were made with the filtrates of young, unautolyzed, broth cultures of Type II pneumococci which contain the S substance and no other known serologically reactive constituent. The fact that potent antiyeast sera invariably precipitated these filtrates with the formation of the compact disc characteristic of S-anti-S precipitates indicates that the same sera would likewise precipitate solutions of the purified carbohydrate derived from Type II pneumococci. While tests with the purified substances would be desirable, one may conclude that in all probability the serological relationships evidenced between the yeast and the Pneumococcus in both the antiyeast and the antibacterial sera are due to reactions between anti-S antibodies and the specific polysaccharides of this variety of yeast and of the Type II variety of Pneumococcus. In view of the usual dependence of immunological relationship upon chemical structure, there must be some chemical likeness between the S substances of these two kinds of microorganisms. The possibility of chemical relationship between the S substances derived from the yeast and the Type II bacteria is particularly interesting since pure solutions of both these polysaccharides have been studied chemically: that of the yeast by Mueller (2), and that of the Pneumococcus by Heidelberger and Avery (3).

The relationship of the yeast to the Type II bacteria is apparently an example of heterogenetic specificity, like that of the Type II pneumococcus to some strains of the Friedländer bacillus (4). The present example is particularly interesting, however, because of the obviously distant genetic relationship of the groups of microorganisms in which the immunologically related strains occur; the one belonging to the Class of *Schizomycetes*, pathogenic and rarely found outside of the animal body; the other belonging to the Class of *Ascomycetes*, non-pathogenic and leading a free existence in nature. The more complete knowledge that is being obtained by chemical studies of the specific antigenic constituents of bacteria is furnishing further demon-

strations of the fundamental principle stated by Wells (5) in his early studies on the plant proteins, that "specificity . . . . is determined by the chemical structure . . . . rather than by the biological origin" of the reacting substances. Since the cells of each plant or animal contain a number of different antigens, there naturally are a number of possibilities for likenesses in the chemical structure of some one of the various cell constituents of phylogenetically unrelated forms of life. Frequently these chemical likenesses may be biologically fortuitous (6) and the consequent serological relationship should be considered an "accident" rather than an index of genetic relationship.

This is obviously the case with the described serological relationship between the yeast and the Type II pneumococcus, where from the standpoint of S-anti-S reactions alone, the Type II pneumococcus has more in common with a particular member of the Class of *Ascomycetes* than with the Type I and Type III varieties of its own species. The latter evidence their real relationship to Type II pneumococci by their mutual P-anti-P reactions, for the P is apparently the genetically significant antigenic constituent of Pneumococcus.

SUMMARY.

The paper reports evidence of an immunological relationship between one variety of *Saccharomyces cerevisiæ* and the Type II variety of *Diplococcus pneumoniæ* (Pneumococcus). The most convincing data consisted of the reactions of the Type II bacteria with potent antiyeast serum which agglutinated, and protected mice against these pneumococci as well as the average antiserum obtained by immunization of rabbits with Type II bacteria themselves. The reactivity of the antiyeast serum is strictly specific to the Type II variety of Pneumococcus in the sense that it is entirely devoid of antibodies reactive with Type I or III. The results of absorption experiments with both the antiyeast (rabbit) serum and the anti-Type II (horse) serum were the same as those usually obtained in analogous experiments with immunologically related, but not identical, kinds of bacteria.

The immunological relationship of the yeast and the Type II pneumococcus is apparently based upon S-anti-S reactions. It repre-

sents an example of heterogenetic specificity which is of particular interest because of the wide genetic separation of the pathogenic schizomycete and the saprophytic ascomycete.

Data on the individual irregularity in the yeast-agglutinating capacity of serum from non-immunized or "normal" rabbits are presented as experimental facts.

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AN EXPERIMENTAL STUDY OF THE ETIOLOGY OF CHICKEN SARCOMA I (ROUS).

By W. E. GYE, M.D., and J. HOWARD MUELLER, Ph.D.

*(From the Department of Bacteriology and Immunity, Harvard University Medical School, and the Cancer Commission of Harvard University, Boston.)**

(Received for publication, October 11, 1928.)

One of us (Gye) has published evidence (1) which indicates that the Rous chicken sarcoma filtrate contains two essential elements, one, a non-living substance, carrying the property of specifically affecting certain cells in the fowl so as to render them susceptible to infection with the second substance, a living virus, which stimulates them to rapid growth in such a way that a tumor is produced. This tumor is identical with the original Rous tumor, and from it, in turn, filtrates are obtainable which contain the same two factors. It also appears that the virus is present in many mammalian tumors, including human carcinoma, thus bringing human cancer into direct relation with the filtrable chicken tumor.

The difficulties which have been experienced by workers in other laboratories in repeating these experiments, and the conviction of one of us (Mueller (2)) that it would be practically impossible to disprove this theory by negative experiments, have led to the joint repetition of the work which is to be here described.

Before proceeding to a description of the experiments, certain general statements must be made in connection with the methods employed. These have been developed entirely by the work in England, and many of them have not hitherto been explained in detail (3). The reasons underlying some of them are not clear, and it must be recognized that in these cases it has been found empirically that they are more likely to lead to satisfactory results than are other forms of technic.

* The expenses of this joint investigation were defrayed by the Harvard Cancer Commission and by the British Medical Research Council; and the experiments were carried out in the Department of Bacteriology of the Harvard Medical School, the authors working together.

Preparation of Filtrates.

It is essential for satisfactory results that filtrates of the chicken tumor which are to be used should be as highly potent as possible, and that they should be entirely cell-free and water-clear.

The color varies from yellow to pink depending on the amount of blood present in the tumor tissue. It has been shown by one of us with Andrewes (4) that the filtrability of the Rous sarcoma may be tremendously variable, reaching a point at times when it is not possible to obtain filtrates which are at all infectious. All stages of potency may be exhibited up to filtrates which will infect in a quantity of 0.001 cc. The reason for this variation is not known, and it is one of the most annoying factors in carrying out the experiments. To guard against extreme variations as far as possible, it is best to use for the preparation of filtrates, tumors which have developed as a result of the inoculation of cells, rather than of filtrates, and only such tumors as grow quickly to a considerable size. "Healthy" tissue from such tumors should be minced and ground thoroughly with clean, neutral sand,[1] in a mortar, and then suspended in salt solution in the proportion of about 8 cc. minced tumor to 200 cc. saline. This should be filtered by gravity or gentle suction through layers of paper pulp and sand in a form of filter tube described elsewhere (4). If the first filtrate is not practically clear, it must be refiltered through a similar filter until all traces of opalescence are removed. It is probable that turbid "sand-filtrates" are responsible for some of the failures which have been reported in attempting to repeat experiments of this type. It is obvious that if large particles of cells are present, or whole cells, virus which may be enclosed in them is protected from the action of added reagents, and may survive much longer than that which is not so situated. The reaction of such filtrates should not vary widely from pH 7.4 For most of the experiments it is then desirable to pass the sand-pulp filtrate through a Mandler or medium Berkefeld filter. The reaction of the final candle filtrate should not have changed. It may be noted that vigorous agitation of the filtrates, either by shaking or through foaming during filtration from excessive suction, will frequently lead to the formation of a coagulum, which may defeat the purpose of the experiment.

Preparation of Specific Factor.

It is probable that a substance which carries, perhaps in a single molecule, the delicate specificity not only of the species, but also of a

[1] If the sand is not clean or shows a reaction on either side of neutrality it will interfere markedly with the reaction of the filtrates, and in many of the experiments which are to be described, coarse carborundum was substituted, and appears to be perfectly satisfactory.

particular type of cell, must be readily susceptible to injury with loss of its biological properties. On the other hand a living virus so widespread in nature that it adapts itself equally to the chicken, the rat, the mouse and man, may conceivably be much more resistant to mild deleterious influences. In any case, the apparent difficulty in carrying out these experiments lies in destroying the virus without at the same time altering the specific factor. To accomplish this with an antiseptic it is essential (*a*) that the reagent shall not produce a precipitate in filtrates which might lead to mechanical protection of the virus or to direct change in the specific factor, and (*b*) that the antiseptic be removed readily, or, if this is impracticable, be so slow in its action that virus added later is not destroyed immediately.

In the experiments first reported, chloroform was the antiseptic used. More recently, acriflavine has been found to act more regularly, but slowly, and its action is affected by several considerations.

In the first place, filtrates of the Rous sarcoma lose their infectivity rather quickly at 37°. The exact time varies with the potency of the particular filtrate which is unknown at the time of preparation. Addition of 10 per cent serum, and incubation in an anaerobic jar, greatly retard this loss. Moreover serum seems to play some part in the germicidal action of acriflavine. It appears, then, that a dilution of 1:10,000 acriflavine in tumor filtrates to which has been added 10 per cent of *fresh* horse serum will usually inactivate the filtrate after 24 hours anaerobic incubation at 37°. The horse blood must have been collected not more than 24 hours previously, and allowed to clot and separate in the ice chest. It must be clear, otherwise it should be centrifuged or passed through a Berkefeld or Chamberland filter. No precipitate should form in this mixture during the incubation. The acriflavine, which is strongly acid (the hydrochloride), may be neutralized with NaOH but it is not certain how much difference this makes. Old or heated serum appears to be unsuitable. An alternative method for securing an acriflavine specific factor is to add ½ cc. of minced Rous tumor to each of several tubes containing 5 cc. Hartley-KCl broth plus 1 cc. fresh horse serum plus 2 cc. of a 1:500 acriflavine solution. The tumor tissue must sink to the bottom of the tubes, which are then incubated 24 hours at 37° in an anaerobic tin. The supernatant fluid is carefully removed, centrifuged if not perfectly clear and used as specific factor. The results are rather less regular than with the filtrate method. In some of the experiments to be described in this paper, fresh rabbit serum was used. There are not sufficient data available to state whether its use is to be recommended.

In connection with the use of chloroform the autoinactivation of filtrates at 37° explains many failures. Not infrequently, and particularly in filtrates which are not highly potent, 3 hours incubation at 37° with no addition of chloroform leads to total loss of infectivity. This change is considerably diminished by HCN in a dilution of 1:10,000 or less. Consequently the chloroform technic has been modified by adding this dilution of HCN (made freshly by neutralizing KCN with HCl) to the filtrate before the chloroform is run in. In some cases mixing the chloroform with the filtrate causes the formation of a precipitate, and for that reason the method which has now been adopted is to leave the chloroform to diffuse through the contents of the tube during incubation without actually mixing. The tube is closed with a rubber stopper to avoid loss through evaporation. At the end of the period of incubation the fluid is transferred to a small flask, and the chloroform vapor removed with a vacuum pump.

Virus Preparation.

Primary cultures of most mammalian tumors in Hartley-KCl-serum broth, grown anaerobically, have been found suitable.

It may be noted that Mouse Sarcoma 37 has never been found to yield a satisfactory virus. A slow growing, and therefore non-filtrable Rous tumor grown aerobically provides an excellent virus. The optimal time of incubation is 3 to 5 days. 2 day cultures are never active, whereas 24 hour cultures may be used provided they are first heated to 55° for 18 minutes. The reason for this heating, as well as for difference in action at various times, is not apparent, but is perhaps connected with some life cycle of the virus. If such cultures are mixed in equal volumes with specific factor preparations, activity is not always, or even usually, restored, but if a series of dilutions of the virus preparation in saline is made 1:4, 1:16 and 1:64, and these dilutions, as well the undilute virus, mixed with specific factor, the chances for reactivation are greater. It would seem that 1:16 or 1:64 is the most regularly effective concentration; greater dilutions fail. In suitable experiments where reactivation takes place with two or more dilutions the results are regular in that at the optimum concentration of virus a large tumor is produced while the others are progressively smaller as the dilution departs from the optimum. The reason for this empirical fact remains obscure.

Another type of virus preparation depends upon the observation that the specific factor in the Rous tumor is more readily destroyed by heating than is the virus. If 3 cc. lots of candle filtrate of the Rous

tumor, containing 10 per cent serum, are heated to 55° for 18 minutes, the ability to infect is lost, whereas it remains able to reactivate a suitable specific factor.

General Considerations.

It must be borne in mind that the great difficulties encountered in such experiments as these make it unlikely that 100 per cent success can be obtained. One has to contend with (*a*) extreme variability in potency of tumor filtrates; (*b*) the unknown part played by the horse serum, which must be fresh, and which seems to vary greatly from different sources; (*c*) differences in filtrates which occasionally lead to precipitate production by the acriflavine; (*d*) the peculiar properties of the virus; (*e*) considerable variation in susceptibility of chickens to the attenuated agent of the tumor; and probably others which are less well defined. For example it is not impossible that the tumor virus, being widely distributed in nature, may be found in the air, and especially so in laboratories which have been used for tumor work. Again, since chickens not infrequently develop spontaneous tumors, some individuals may harbor the virus in their bodies. Occasional falsely positive controls, therefore, need not be surprising. In any case, great pains should be taken to prevent specific factor preparations from becoming contaminated from the hands or the air.

Where satisfactory reactivation takes place, it must not be supposed that the full original infectivity of the filtrate is restored. The specific factor is always greatly weakened by either of the methods employed, and tumors from mixtures of virus and specific factor do not usually appear in less than 3 or 4 weeks.

In most of the experiments to be described, there were included controls of chick embryo cultures (5), not inoculated with any tumor material, mixed with specific factor instead of virus.

It may be repeated that the foregoing details of technic had been established previous to the joint experiments now to be reported. Experimental proof of them will appear separately and will not be included here. Since the acriflavine technic was considered the most regularly satisfactory, most of the experiments are of this type. Since one of us has insisted in a previous communication (2) that it is essential to present all the experiments carried out in sequence, this plan is

here followed, although for the sake of brevity this is done in the form of a table, and only those experiments marked with an asterisk are considered in any detail. A few attempts were made to demonstrate two factors by means of chloroform specific factor. In every case these experiments were completely negative. Allowing the chloroform to diffuse through the filtrate without mixing failed in each case to inactivate, whereas when it was mixed by means of a capillary pipette, no tumors developed in either controls or mixtures. These experiments are consequently omitted.

Acriflavine Experiments.

Date	General results
* Oct. 18, 1927	1. Controls negative, one tumor from mixtures. 2. Controls and mixtures negative.
* Oct. 25, 1927	Controls negative, one mixture positive.
* Oct. 26, 1927	Controls negative, two mixtures positive.
* Nov. 7, 1927	1. One control positive, all mixtures negative. 2. Controls and mixtures all positive.
Nov. 12, 1927	Controls and mixtures positive.
Nov. 13, 1927	Controls and mixtures negative.
* Nov. 19, 1927	Some controls and some mixtures positive.
* Nov. 26, 1927	One control and three mixtures positive.
Nov. 28, 1927	Three out of four controls and five out of six mixtures positive.
Dec. 1, 1927	Controls and mixtures negative.
* Dec. 2, 1927	Controls negative,half of mixtures positive.
Dec. 5, 1927	Controls and mixtures negative (filtrate inactive).
* Dec. 7, 1927	Controls negative, one mixture positive.
* Dec. 14, 1927	Controls negative, one mixture positive.
Dec. 16, 1927	Controls and mixtures positive.
Dec. 22, 1927	Controls and mixtures negative (weak filtrate).
Dec. 23, 1927	Controls and mixtures negative (weak filtrate).
* Dec. 30, 1927	Controls negative, two-thirds of mixtures positive.
* Jan. 2, 1928	Controls positive, three mixtures out of four positive.

In describing the acriflavine experiments, the specific factors will be noted simply as having been made by the "acriflavine-filtrate" or "acriflavine-culture" method, referring to the two procedures already described. In every case equal volumes of specific factor and virus, either undilute, or diluted as indicated with saline, were mixed, and 1 cc. of the mixture injected. A trace of pure silica was added to

each inoculum. In all the work, Barred Rock chicks from 6 to 10 weeks old were used. As a rule, six injections were made into each bird, in breasts, legs and wings. In controls, however, usually only a single inoculation was made, this in the breast.

Experiments with Negative Controls and Some Positive Mixtures.

Oct. 18, 1927.

S. F.—Acriflavine-culture specific factor.

The virus preparations were the following:

V_1—Rous sarcoma filtrate plus 10 per cent horse serum, heated 18 minutes at 55°C.

V_2—5 day aerobic culture of a human uterine fibroid in Hartley-KCl-horse serum broth.

V_3—6 day aerobic culture of the Rockefeller rabbit carcinoma.

	V_1			V_2			V_3	
Bird No	1	2	3	4	5	6	7	8
S. F. 1.0 cc	−	−	−	−	−	−	−	−
S. F. ½ cc. + V − ½ cc	−	−	−	−	−	−	−	−
S. F. ½ cc. + V − 1:16 − ½ cc	−	−	−	−	−	−	−	++++
S. F. ½ cc. + V − 1:64 − ½ cc	−	−	−	−	−	−	−	

Oct. 25, 1927.

S. F.—Acriflavine-culture specific factor.

V_1—Supernatant ot Rous sarcoma culture, heated 18 minutes to 55°.

V_2—First subculture of human fibroid, in KCL-serum broth containing chick embryo tissue.

E—Control uninoculated tube of chick embryo.

	V_1				V_2		E	
Bird No	9	10	11	12	13	14	15	16
S. F. 1.0 cc	−	−	−	−	−	Dead Nov.	−	−
S. F. ½ cc. + V−½ cc	−	−	−	−	−	11. No	−	−
S. F. ½ cc. + V − 1:4 − ½ cc					−	tumors	−	−
S. F. ½ cc. + V − 1:16 − ½ cc	−	−	−	++++	−		−	−
S. F. ½ cc. + V − 1:64 − ½ cc	−	−	−		−		−	−

V_1 was controlled separately on Chicks 17 and 18, undiluted and diluted 1:16 and 1:64. No tumor resulted.

Oct. 26.
S. F.—Acriflavine-filtrate specific factor.
V_1—Rous tumor filtrate plus 10 per cent horse serum—heated.
V_2—5 day anaerobic culture of a human carcinoma.

	Controls				V_1		V_2	
Bird No.	19	20	21	22	23	24	25	26
S. F. 2.0 cc.	−	−	−	−				
S. F. ½ cc. + V − ½ cc.					−	+	−	++
S. F. ½ cc. + V − 1:4 − ½ cc.					−	−	−	+++
S. F. ½ cc. + V − 1:16 − ½ cc.					−	−	−	+
S. F. ½ cc. + V − 1:64 − ½ cc.					−	++	−	+
V − ½ cc.					−	−		
V − 1:64 − ½ cc.					−	−		

Dec. 2, 1927.
S. F.—Acriflavine-filrate specific factor.
V_1—Heated Rous sarcoma filtrate.
V_2—Rat 9 carcinoma 24 hour anaerobic culture—heated 18 minutes to 55°.
E—24 hour anaerobic "culture" chick embryo, heated 18 minutes at 55°.

	Controls				E*		V_1		V_2*	
Bird No.	27	28	29	30	31	32	33	34	35	36
S. F. 2.0 cc.	−	−	−	−						
S. F. ½ cc. + V − ½ cc.					++	++	−	−	−	−
S. F. ½ cc. + V − 1:4 − ½ cc.					−	−	−	−	−	−
S. F. ½ cc. + V − 1:16 − ½ cc.					−	−	−	−	−	−
S. F. ½ cc. + V − 1:64 − ½ cc.					−	−	++++	−	−	−
V − ½ cc.							−	−		

* It may be said in connection with this experiment that the tumors of Chicks 31 and 32, while small, had penetrated the abdomen, causing extensive tumor formation here which was infected. It is not improbable that the original material mixed with the specific factor was contaminated. Moreover, there was some question as to whether the E and V_2 preparations might not have been exchanged in setting up the mixtures.

Dec. 7, 1927.

S. F.—Acriflavine-filtrate specific factor.

V_e—5 day anaerobic "culture" of chick embryo.

V_1—5 day anaerobic culture of Rat 9 carcinoma.

	Controls				V_e		V_1	
Bird No.	37	38	39	40	41	42	43	44
S. F. 2.0 cc.	−	−	−	−				
S. F. ½ cc. + V − ½ cc.					−	−	−	++
S. F. ½ cc. + V − 1:4 − ½ cc.					−	−	−	−
S. F. ½ cc. + V − 1:16 − ½ cc.					−	−	−	++
S. F. ½ cc. + V − 1:64 − ½ cc.					−	−	−	−

Dec. 14, 1927.

S. F.—Acriflavine-filtrate specific factor.

V_e—5 day aerobic "culture" of chick embryo.

V_1—7 day anaerobic subculture (first generation) of Rat 9 carcinoma.

V_2—7 day anaerobic subculture (third generation) of rabbit carcinoma.

V_3—24 hour aerobic culture of the Rat 9 carcinoma which through oversight was not previously heated (see earlier remarks on "virus").

V_4—5 day aerobic culture of Mouse 63 carcinoma.

	Controls				V_e		V_1		V_2		V_3		V_4
Bird No.	45	46	47	48	49	50	51	52	53	54	55	56	57	58
S. F. 2.0 cc.	−	−	−	−										
S. F. ½ cc. + saline ½ cc.	−	−	−	−										
S. F. ½ cc. + V − ½ cc.					−	−	−	−	−	−	−	−	−	?*
S. F. ½ cc. + V − 1:4 − ½ cc.					−	−	−	−	−	−	−	−	−	?
S. F. ½ cc. + V − 1:16 − ½ cc.					−	−	−	−	−	−	−	−	−	++++
S. F. ½ cc. + V − 1:64 − ½ cc.					−	−	−	−	−	−	−	−	−	−

* A tumor was present in the peritoneum, apparently starting near the keel bone, but from which side was not clear.

Dec. 30, 1927.

S. F.—Acriflavine-filtrate specific factor.

V_e—3 day aerobic chick embryo "culture."

V_1—3 day aerobic Rat 9 culture.

V_2—Rous filtrate + 10 per cent horse serum heated 18 minutes to 55°.

	Controls				V_e		V_1		V_2	
Bird No.	59	60	61	62	63	64	65	66	67	68
S. F. 2.0 cc.	−	†	−	−						
S. F. ½ cc. + V − ½ cc.					−	−	−	−	−	−
S. F. ½ cc. + V − 1:4 − ½ cc.					−	−	−	−	−	−
S. F. ½ cc. + V − 1:16 − ½ cc.					−	−	++	−	−	−
S. F. ½ cc. + V − 1:64 − ½ cc.					−	++++	−	++	−	+++
V_2 − ½ cc.									−	−

† Dead Jan. 9—no tumor.

Experiment with a Positive Control and All Mixtures Negative.

Nov. 7, 1927.

S. F.—Acriflavine-filtrate specific factor.

V_1—4 day anaerobic culture of Mouse 63 carcinoma.

V_2—Subculture of Rous tumor (4th generation), 0.25 cc. of 3rd subculture added to tube of serum-broth without embryo.

V_3—Subculture of human breast carcinoma, 4th generation, made in same way as V_2.

	V_1			V_2		V_3	
Bird No.	69	70	71	72	73	74	75
S. F. 1.0 cc.	−	−	++++	−	−	−	−
S. F. ½ cc. + V − ½ cc.	−	−	−	−	−	−	−
S. F. ½ cc. + V − 1:16 − ½ cc.	−	−	−				
S. F. ½ cc. + V − 1:64 − ½ cc.	−	−	−				

Experiments with One or More Positive Controls and Mixtures.

Nov. 19, 1927.

S. F.—Acriflavine-filtrate specific factor, final concentration of 1:7000 acriflavine used in this experiment.

V_1—3 day anaerobic culture of Mouse Carcinoma 63.

V_2—3 day anaerobic culture of rabbit carcinoma.

	Controls				V_1		V_2	
Bird No.	76	77	78	79	80	81	82	83
S. F. 5.0 cc.	−	†						
S. F. 2.0 cc.			++++	++				
S. F. ½ cc. + V − ½ cc.					+	+++	+	+++
S. F. ½ cc. + V − 1:4 − ½ cc.					++	+++	−	−
S. F. ½ cc. + V − 1:16 − ½ cc.					++++	++++	−	+
S. F. ½ cc. + V − 1:64 − ½ cc.					+++	+++	++++	+++
V − 1 cc.					−	−	−	−

† Missing, probably dead and accidentally discarded before making first record.

Nov. 26, 1927.

S. F.—Acriflavine-filtrate specific factor.

E_1—Sand-pulp filtrate of chick embryo+10 per cent horse serum, heated 55° for 18 minutes.

V_1—Similarly heated Rous filtrate plus horse serum.

E_2—6 day "culture" of chick embryo.

V_2—6 day culture of rabbit carcinoma.

The mixtures of S. F. with E_2 and V_2 were given on opposite sides of the same chicks.

	Controls			E_1			V_1			E_2			V_2		
Bird No.	84	85	86	87	88	89	90	91	92	93	94	95	93	94	95
S. F. 2 cc.	−	++++	−												
S. F. ½ cc. + V − ½ cc.				−	−	−	−	−	−	−	−	−	−	−	−
S. F. ½ cc. + V − 1:4 − ½ cc.				−	−	−	−	+	−	−	++++	−	−	−	−
S. F. ½ cc. + V − 1:16 − ½ cc.				−	−	−	−	−	−	−	++	−	−	−	−
S. F. ½ cc. + V − 1:64 − ½ cc.				++++	−	−	−	−	++++						
V_1 ½ cc.							−	−	−						
V_1 − 1:16 − ½ cc.							−	−	−						

Jan. 2, 1928.
S. F.—Acriflavine-filtrate specific factor.
V_e—5 day aerobic "culture" of chick embryo.
V_1—4 day aerobic culture of rabbit carcinoma.

	Controls				V_e		V_1	
Bird No	96	97	98	99	100	101	102	103
S. F. 2.0 cc	++	+	†	++++				
S. F. ½ cc. + V − ½ cc					−	−	±	−
S. F. ½ cc. + V − 1:4 − ½ cc					−	−	−	±
S. F. ½ cc. + V − 1:16 − ½ cc					−	++++	−	++
S. F. ½ cc. + V − 1:64 − ½ cc					−	−	−	−

† Chick 98 died Jan. 9 with no tumor.

DISCUSSION (Mueller).

To know how to deal fairly with results of this type is obviously an exceedngly difficult matter. The method used is directed at the production of an inactive specific factor, and this end seems to have been reached or approached in a number of the experiments cited. Those experiments in which practically all sites inoculated were positive, have been arbitrarily excluded. Such experiments are clearly defective, and to include them would be scarcely reasonable.

On the other hand, if this discrimination is permissible, would it perhaps be wiser to exclude all those experiments in which any positive controls appeared, since in them obviously the inactivation has failed, unless one assumes the possibility of contamination from the air or some other source? This has not been done because it appears that the probable explanation of these results, while it is suggested in some of those with negative controls, receives further support from the others.

Moreover, it is from a consideration of results as a whole that one must draw conclusions in this type of experiment. The writer has already stated in his earlier paper dealing with the chloroform technic, that in addition to a considerable variation in susceptibility on the part of individual chickens, there is also a marked local difference in reaction in different sites on the same chicken. It is clear that, within certain limits, the amount of a Rous sarcoma filtrate governs time of take, rate of growth and size of tumor, and that there is thus a degree

of proportionality between the size of tumor produced and the infecting dose of filtrate. But once an extreme degree of attenuation has been reached by either chloroform, serum-acriflavine or probably also simple dilution, it would perhaps be surprising if a local difference in result were not found from injections of equal amounts. There may be factors which come into play, such as trauma, hemorrhage, etc., which are entirely beyond control and which may play a decisive part in the result with these small doses of attenuated material.

It becomes important to determine whether a control injection of 2.0 cc. of specific factor is, on the average, four times as likely to infect, where a considerable number of injections are given, as 0.5 cc. doses. There is one significant indication in the experiment of November 19. Two controls received 5 cc. and two, 2 cc. Both the latter as well as all the mixture chicks developed tumors. One of the 5 cc. control chicks died too soon to obtain a result and the other remained negative. Was this one an unusually resistant chick, or did the amount of acriflavine injected into the tissue play some inhibitory rôle? In the latter case, the usual procedure of four 2.0 cc. control injections would become still more unreliable.

If now, the apparently satisfactory experiments are reviewed with this point in mind, a number of things become clear. First, the "reactivation" with virus, if there be one, is a very feeble affair. There is only occasional indication of it. The tumors develop slowly and while it has seemed best in the interest of brevity to omit dates and consecutive observations, it may be said in general that those tumors which do develop do so only after a considerably longer interval than is required after the injection of a moderately active filtrate and do not differ materially one way or the other in rate of growth from tumors produced by specific factor alone. In the second place, as a rule only one-fourth to one-sixth as many sites are injected with specific factor controls as with mixtures. By the laws of chance there should be a greater number of "takes" registered in the latter. Thirdly, there are a reasonable proportion of positive results in the "embryo culture" control mixtures. And finally, it is rather the rule than otherwise, that where both chicks injected with a single mixture of specific factor and virus develop tumors, one dilution of virus is positive in one, and a different one in the second. For example in the experiment of Dec.

30, one tumor appeared with the 1:16 dilution and the other with the 1:64. The tumors were similar in size. Obviously both chicks were susceptible in about the same degree. Also obviously both mixtures contained infectious material. Therefore, the result must be accidental and without significance. As to the supposed proportionality between dilution of virus and ability to reactivate, there is surely nothing to bear it out in these results. The whole matter is so irregular that chance seems to be the only adequate explanation.

The writer does not wish to put this forward as a definite conclusion without further experimental evidence. He has therefore carried out several experiments, using exactly the same technic with the exception of filtering through rather less paper pulp in a filter so prepared that leakage around the edges was not possible. (It may be stated that in filter tubes prepared as Gye and Andrewes originally described them, fluid will almost always, after a time, find its way down between the glass and the pulp, producing a turbid filtrate which has to be passed through a second filter to clear it, with proportional loss of infectiousness.) The filtrates used in these experiments were of a somewhat higher potency than those obtained in the collaborative experiments with Gye, but were uniformly clear and sparkling.

Instead of controlling as in the experiments already described, four chicks were used as controls, each injected with acriflavine-filtrate specific factor in four sites (both breasts and legs). Two were given 2.0 cc. undiluted specific factor in one breast, 1.0 cc. diluted with 1.0 cc. normal saline in the other breast and $\frac{1}{2}$ cc. + $\frac{1}{2}$ cc. saline in each leg. The other two received $\frac{1}{2}$ cc. specific factor diluted with $1\frac{1}{2}$ cc. saline in each breast and $\frac{1}{2}$ cc. + $\frac{1}{2}$ cc. saline in each leg.

It will be sufficient to tabulate the results of the control inoculations, since the mixtures which were made,—in each case two "viruses" and one "embryo control" on two chicks each,—turned out very much as did those of the earlier experiments and as one would expect from the controls. If anything, fewer tumors were obtained from mixtures than from controls.

The last two experiments quoted, Jan. 18 and Feb. 2, were the most nearly ideal since fewer tumors were obtained in them than in the others. It will be observed that while three out of four chicks in each experiment developed one or more tumors, in the experiment

	Jan. 13				Jan. 16				Jan. 17				Jan. 18				Feb. 2			
Chick No.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
2 cc. S.F. (left breast)	++	−			++++	++++			−	+++			−	++++			++	−		
1 cc. S.F. + 1 cc. saline (right breast)	++	−			++++	−			++	+			−	+			−	−		
½ cc. S.F. + ½ cc. saline (left leg)	++++	−	+++	+++	+++	+	++++	++++	+++	++++	++	−	−	−	−	++	−	+	−	−
½ cc. S.F. + ½ cc. saline (right leg)	−	++++	++	+++	+++	−	++++	+++	−	+	++++	++	−	++	−	++	−	++++	−	++++
½ cc. S.F. + 1½ cc. saline (left breast)			−	++			++++	++++			+++	++++			+	++++			−	−
½ cc. S.F. + 1½ cc. saline (right breast)			++	++			++++	++++			++	++++			−	−			−	−

just mentioned, only one small tumor, and in the second, no tumors at all developed in the right breast of any of them (the site ordinarily used for a single control). Of course, this was pure accident, but indicates the sort of unusual combinations that turn up in this work. Had only the right breasts of the chickens used as controls in the last experiment been inoculated, one might have obtained a completely negative control series, and since a number of tumors developed from various mixtures employed in the experiment, the effect would have been that of a definite "reactivation" of the specific factor.

We now have a reasonable explanation which is supported not only by indirect evidence from the experiments carried out jointly with Gye, but also by direct experimental evidence, which we venture to say can be duplicated readily. Obviously this does not disprove Gye's hypothesis, nor does it even permit definite statement that all the experiments quoted above were accidental. In some of them a possible,—though weak,—mechanism for reactivation may have actually operated. For example in the experiment of December 14, the controls were done better than in the other experiments, and in addition, V_e, V_1 and V_2 were not "optimal" virus preparations, but were at least thorough embryo controls, and V_3 was not expected to be suitable since the preliminary heating to 55° was forgotten. There is thus left V_4, which was a 5 day culture of Mouse Carcinoma 63, an entirely satisfactory "virus," and the only tumors of the entire experiment were produced in one of the two chickens injected with it. There is no possible way to decide whether or not this was an accident.

To sum up, one may say that in general the form of protocol recommended by Gye in attempting to establish two factors in the Rous sarcoma is unsuitable. The very real local variation in susceptibility is neglected, and the facts do not bear out the assumption that four times the dose of an attenuated filtrate is regularly four times as infectious as a single dose. Both direct and indirect evidence is given to show that in fact this is not the case, but that unknown local conditions, and perhaps the concentration of acriflavine in the filtrate, are of much greater importance. We repeat that this is not disproof of Gye's hypothesis, but insist that in none of the acriflavine work carried out in this laboratory has there been any convincing evidence in its favor.

DISCUSSION (Gye).

3 years ago, in a preliminary notice of a study of the Rous sarcoma (1), evidence was brought forward which in my judgment showed that the induction of a new tumour by a cell-free filtrate depends upon the combined action of two things contained in a filtrate; one which is most easily interpreted as being a filtrable virus, the other a specific aggressin. One of the methods of demonstrating this was to destroy the activity of a tumour filtrate by the action of chloroform and to restore its activity again by the addition of an extract of tumour which had been obtained by allowing tumour tissue to stand in a serum medium for many days at incubator temperature. It was pointed out in the preliminary paper that often chloroform fails to inactivate an extract and that frequently the extract obtained by long incubation used for reactivation is itself active; further, it was noted that sometimes when both chloroform-treated extract and incubated extract are inactive the mixtures are inactive. Nevertheless, the successful experiments were so plain in their meaning that it was impossible not to believe that the regular demonstration of this dual nature of the Rous infective material depends merely upon the discovery of a suitable technique.

The findings given in my preliminary paper have, for the most part, been rejected by other workers. During the past 3 years I have endeavoured to find a more suitable technique, and have failed. The best available so far is as follows:

For the preparation of what I shall call, and believe to be, "virus," a cell-free extract, to which horse serum to the extent of 10 per cent has been added, is heated to 55°C. for 18 minutes. This treatment invariably inactivates the filtrate, the inactivation being due to the destruction of the specific aggressin and not "virus." Virus is left.

The inactivation of a filtrate by means of an antiseptic is a different matter. Chloroform inactivates so irregularly as to be exasperating in a long series of experiments. Moreover, since mere incubation of a filtrate tends to destroy activity in a few hours and sometimes actually does so completely, it is obviously important to protect the filtrate against such loss. This may be achieved by the addition of serum to the filtrate. Chloroform is even less effective in the presence of a serum. For these reasons acriflavine, which is reputed to act in the presence of serum, was chosen as the antiseptic. After much experimentation it was found that acri-

flavine is effective only in the presence of fresh serum—horse serum was used in all my experiments—but then not always.

The method then which had given best results in my hands was as follows:

Fresh horse serum to the extent of 10 per cent is added to a cell-free extract. To a portion of this mixture acriflavine is added to give a final concentration of 1:10,000. The mixture is incubated anaerobically for 24 hours in a McIntosh-Fildes jar. Reactivation is obtained by adding diluted heat-inactivated extract.

In 1927 I was invited by the Cancer Commission of Harvard University to visit Dr. Zinsser's laboratory in order to compare technical methods with Dr. Mueller who had failed to confirm my published observations. During October and November we worked together and the results of our collaboration are given in this paper. The proportion of experiments which give support to my contentions is much smaller than I expected, judging from a longer series which had already been carried out in my laboratory in London. This longer series of similar experiments will be published during the present year, together with a discussion of alternative interpretations. The many difficulties encountered in all this work have made earlier publication impossible.

Two comments upon the action of acriflavine on filtrates of the Rous sarcoma may be useful, and as they have been made perfectly certain by extensive experimentation, may be stated categorically:

1. Acriflavine in itself is a very feeble antiseptic towards the virus of the filtrable fowl sarcoma. Proof of this statement has been obtained by allowing acriflavine to act upon filtrates in which cysteine has been dissolved to prevent loss of infectivity by oxidation (6). Under such circumstances it is impossible to sterilize (in 24 hours), with any possible concentration of acriflavine, a very active filtrate. Feebly active extracts are rendered inert in 24 hours by dilution of acriflavine of 1:10,000. In these experiments the acriflavine is neutralised before adding to tumour extracts, otherwise precipitates are formed which nullify the experiment.

2. When the infectivity of a Rous tumour extract is destroyed by the action of acriflavine in the presence of fresh horse serum, the result is governed by the viricidal action of the serum, acriflavine acting in a merely supplementary way.

My colleague, Dr. W. J. Purdy, and myself have investigated this action thoroughly. We have compared as exactly as possible the

lethal effect of fresh serum plus acriflavine upon the virus of bovine pleuropneumonia and the inactivating effect of the same combination upon Rous tumour filtrates, and have found that they follow the same rules. The natural viricidal properties of a given fresh serum dominate the final result of an experiment. The details of experiments which support this statement are now in preparation.

It will be seen from these comments that the experiments which Dr. Mueller and I report in this paper are more complex than might at first glance be supposed.

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BLOOD REGENERATION IN SEVERE ANEMIA.

XIV. A Liver Fraction Potent in Pernicious Anemia Fed Alone and Combined with Whole Liver, Liver Ash and Fresh Bile.

By F. S. ROBSCHEIT-ROBBINS and G. H. WHIPPLE.

(*From the Department of Pathology, The University of Rochester School of Medicine and Dentistry, Rochester, N. Y.*)

(Received for publication, October 25, 1928.)

Liver extracts are of peculiar interest at this time because of the keen clinical interest in the diet therapy of pernicious anemia. The liver fraction which has been studied with greatest care is that known as liver extract, No. 343, N.N.R., prepared by Eli Lilly and Company under the supervision of the Committee on Pernicious Anemia of the Harvard Medical School according to the method of Cohn, Minot, Alles and Salter (1). Through the cordial interest of Dr. Minot and the Harvard Committee we have been able to test this extract in severe secondary experimental anemia due to continued bleeding.

This liver extract No. 343 has been widely tested with favorable results in pernicious anemia. It has been used in a variety of other anemias and the general experience would seem to be that it may act favorably in such anemias as resemble pernicious anemia (for example sprue) but it is relatively inert in the common cases of secondary anemia. Secondary anemias in man are notoriously complex and it would certainly be of interest to test carefully liver fractions in all types of anemia. This discussion has recently been elaborated elsewhere (3, 4).

In general we may say of our experiments with this liver extract that it is relatively inert when compared with whole liver feeding in severe anemia due to hemorrhage in dogs. We may conclude that the liver extract may be 10 to 20 per cent as potent as the whole liver, but individual variations are more conspicuous than with whole liver feeding (Table 141).

We supplemented this liver extract with small amounts of whole liver, liver and apricot ashes and fresh bile to ascertain whether this liver extract might become fully potent when these other factors were added. The results given below show that ash plus this liver extract gives the values expected from the combined effects of the individual ash and liver fractions. Fresh dog bile by itself in diets is inert and it adds nothing to this liver extract diet effect. When we supplement this liver extract with small amounts of whole liver in the diet mixture we observe at times hemoglobin and red cell production in excess of the sum of the expected separate reactions.

The general methods used in these experiments have been described in detail in the first (5) and fifth (6) papers of this series. The *standard bread* used in all these experiments is described as to ingredients and preparation. This bread contains wheat flour, starch, bran, sugar, cod liver oil, canned tomatoes, canned salmon, yeast and a salt mixture. This bread is a complete diet for an adult dog and will maintain a dog in health for long periods of time, if not indefinitely. This ration keeps the hemoglobin production at a very low level, the average being close to 1 to 3 gm. of hemoglobin per week period over and above the unknown red blood cell maintenance factor. There are individual differences and some dogs are constantly high in hemoglobin production on the basal rations as well as on other diets. The *hemoglobin index* described in these papers is a figure obtained by dividing the hemoglobin per cent by the red cell hematocrit in per cent. We believe this figure gives information of value as to the saturation of the red cell stroma with hemoglobin.

It is important to remember that these standard dogs are in a condition of sustained anemia (40 to 50 per cent hemoglobin) month after month, year after year. Some of the dogs have been *continuously anemic* as indicated for 4 years and are reacting quantitatively to various specific diet intakes in the 4th year as they did in the 1st year. The experimental data presented in the tables are taken out of the dog's experimental history which is continuous. The figures given represent the observations made at the *end of any given week* so that the hemoglobin figures noted for the 1st week of bread feeding represent the production of hemoglobin due to 7 days of bread feeding.

We note the importance of a *sustained maximal stimulus to hemoglobin production* in these dogs whose circulating hemoglobin level is maintained at 40 to 50 per cent hemoglobin, or approximately one-third normal for the dog. The surplus hemoglobin removed by bleeding to maintain this base line level is measured accurately and represents the capacity of the dog to form hemoglobin and red cells on the given food intake over and above the unknown red cell replacement factor. These dogs are raised in our kennels and are accustomed to the environment and experimental technic so that they lead normal, active lives undisturbed by the experi-

mental observations, unless some note is made to the contrary. Uniformity of environment is very important and includes heat, ventilation, exercise and isolation to minimize cross-infection.

The precise lot of the liver extract No. 343 used in these experiments on dogs was tested under the supervision of the Harvard Committee in cases of pernicious anemia and was shown to be fully potent. Moreover, all the material used was from a single batch and therefore of uniform constitution and potency throughout all our experiments.

EXPERIMENTAL OBSERVATIONS.

That these anemic dogs show considerable individual variation in reaction to this liver fraction is obvious from an explanation of Table 141. There is no such individual variation to the feeding of whole liver. The first animal (Dog 25–24) presents the average reaction to feeding about 15 gm. of this liver extract No. 343 daily or the amount derived from 500 gm. fresh liver. This 2-week feeding results in the production of about 20 gm. of hemoglobin above the base line level. There is no significant change in the hemoglobin index in any of the three experiments. The second experiment (Dog 24–45) shows a negative reaction to the liver extract fed daily for 2 weeks.

The third experiment (Dog 24–59) shows an unusually high hemoglobin production as the result of daily feeding for 2 weeks of the liver extract derived from 500 gm. of liver. About 40 gm. hemoglobin above the base line is produced during the feeding period combined with the 2nd week of "carry over." This dog during the past several months has reacted with unusual hemoglobin production to the feeding of all meat products. We cannot explain this individual peculiarity but this knowledge enables us to discount this unusual figure.

In some instances the liver extract was tested alone and combined with whole liver or liver ash on the same animal (Dog 21–67, Table 142). These experiments in Table 142 were not continuous but other diet experiments intervened between these three distinct experimental periods. This dog shows an average reaction to the liver extract alone (derived from 400 gm. whole liver) fed daily for 2 weeks. This raises hemoglobin production only 13 gm. above the base line level. When this same amount of liver extract is supplemented by a small dose (100 gm.) of whole liver we note a striking rise in hemoglobin production to a total of about 65 gm. per 2-week feeding period. We

TABLE 141.

Liver Extract No. 343, N. N. R. Derived from 500 Gm. Liver Daily in Diet.

Diet periods 1 wk. each	Food cons.	Wt.	Plasma vol.	RBC	Color index	Hb. index	RBC hemat.	Blood hb. level	Hb. removed bled
Food, *gm. per day*	%	*kg.*	*cc.*	*mil.*			%	%	*gm.*
Dog 25–24. Coach, male, adult									
Br. 400, salm. 75, Kl. 25....	100	12.9	816	4,2	0.52	2.00	22.1	44	1.3
* Liver ext. 500.....	100	12.9	829	5,3	0.48	2.11	22.4	47	13.0
* Liver ext. 500.....	100	12.9	803	5,6	0.44	1.96	24.9	49	1.4
Br. 400, salm. 75, Kl. 25....	100	13.2	798	6,2	0.44	2.04	24.5	50	13.8
Br. 425, salm. 75, Kl. 25....	100	12.9	816	5,2	0.47	2.18	22.4	49	1.5
Dog 24–45. Bull, female, adult									
Br. 350, salm. 75.....	100	18.9	1080	4,8	0.41	2.14	18.5	40	1.2
Br. 350, salm. 75.....	100	18.9	1050	5,3	0.42	2.21	20.0	44	1.3
† Liver ext. 500.....	100	19.0	1115	5,8	0.42	2.16	22.7	49	1.3
† Liver ext. 500...	100	19.3	1100	5,5	0.41	2.13	21.4	46	1.3
Br. 350, salm. 100.....	100	19.3	1116	5,6	0.39	2.13	20.7	44	1.4
Dog 24–59. Bull, male, young adult									
Br. 450, salm. 100.....	100	15.9	1010	4,3	0.49	1.91	21.9	42	1.3
Br. 500, salm. 100.....	100	16.1	1022	4,4	0.47	1.80	22.7	41	1.2
‡ Liver ext. 500.....	100	15.8	1036	5,8	0.48	1.95	25.9	50	16.6
‡ Liver ext. 500.....	100	15.9	1060	5,5	0.48	1.99	25.2	50	15.8
Br. 450, salm. 75.....	100	16.0	1000	5,4	0.43	1.90	24.5	47	1.3
Br. 450, salm. 75.....	100	16.1	1020	5,8	0.47	1.99	22.9	46	15.1
Br. 450, salm. 75.....	100	16.0	1000	5,7	0.39	1.85	23.7	44	2.3

* Liver fraction supplemented by bread 375, Klim 25 daily.

† Liver fraction supplemented by bread 325, salmon 100, Klim 25 daily.

‡ Liver fraction supplemented by bread 400, salmon 75, daily.

note a total bleeding of 74 gm. hemoglobin above the base line output on bread alone, but the initial blood hemoglobin level was 50 per cent and the level at the end was 41 per cent. This difference of 9 per cent

TABLE 142.

Liver Extract No. 343, N. N. R. Combined with Whole Liver and Liver Ash.

Dog 21–67. Bull, male, adult.

Diet periods 1 wk. each	Food cons.	Wt.	Plasma vol.	RBC	Color index	Hb. index	RBC hemat.	Blood hb. level	Hb. removed bled
Food, *gm. per day*	%	*kg.*	*cc.*	*mil.*			%	%	*gm.*
Br. 300, salm. 100, Kl. 25...	94	12.4	634	3,5	0.63	2.27	19.2	44	1.4
* Liver ext. 400.....	87	12.2	600	3,8	0.65	2.31	21.4	49	1.5
* Liver ext. 400.....	95	12.3	620	4,4	0.67	2.33	18.7	43	14.9
Br. 225, salm. 100, Kl. 25...	95	12.2	634	3,8	0.57	2.29	18.9	43	1.2
Br. 225, salm. 100, Kl. 25...	96	12.4	662	3,6	0.69	2.26	22.2	50	1.3
† Liver ext. 400, liver 100...	99	11.9	580	5,3	0.73	2.26	23.1	52	32.7
† Liver ext. 400, liver 100...	100	12.3	742	5,1	0.76	2.48	19.1	47	33.7
Br. 250, salm. 100, Kl. 25...	100	12.3	628	3,8	0.71	2.31	18.9	44	12.7
Br. 275, salm. 100, Kl. 25...	95	12.4	706	3,0	0.68	2.11	19.4	41	1.3
Br. 300, salm. 100, Kl. 25...	89	12.3	662	3,3	0.70	2.24	20.6	46	1.6
‡ Liver ext. 500, liver ash 500.....	90	12.4	543	5,4	0.71	2.31	21.6	50	29.5
‡ Liver ext. 500, liver ash 500.....	98	11.9	566	4,7	0.78	2.31	19.2	44	30.9
Br. 300, salm. 100, Kl. 25...	98	12.5	623	3,4	0.75	2.13	15.4	33	12.1
Br. 300, salm. 100, Kl. 25...	90	12.4	634	3,2	0.61	2.24	17.4	39	1.4

* Liver fraction supplemented by bread 300, salmon 100, Klim 25 daily.

† Liver fraction plus whole liver supplemented by bread 200 daily.

‡ Liver fraction plus liver ash supplemented by bread 200 daily.

hemoglobin means that about 9 to 10 gm. of the hemoglobin were removed which cannot be credited to this diet period. The net amount of hemoglobin production—about 65 gm.—is to be credited to this diet intake of salmon bread plus the liver extract plus 100 gm.

TABLE 143.

Liver Extract No. 343, N. N. R. Fed with Small Amounts of Cooked Whole Liver.

Diet periods 1 wk. each	Food cons.	Wt.	Plasma vol.	RBC	Color index	Hb. index	RBC hemat.	Blood hb. level	Hb. removed bled
Food, *gm. per day*	%	*kg.*	*cc.*	*mil.*			%	%	*gm.*
Dog 24–22. Coach, female, adult									
Br. 300, salm. 75, Kl. 25....	90	12.8	692	5,6	0.42	1.85	25.6	47	1.3
* Liver ext. 500.....	100	12.9	670	5,8	0.38	1.79	23.3	41	2.0
* Liver ext. 500.....	100	12.7	694	5,7	0.42	1.79	26.8	48	1.5
* Liver ext. 500.....	100	13.0	710	6,2	0.45	1.91	25.2	48	15.8
Br. 300, salm. 75, Kl. 25....	100	13.1	794	5,4	0.40	1.84	23.1	43	1.3
Br. 300, salm. 75, Kl. 25....	98	13.0	754	5,2	0.42	1.83	24.1	44	1.3
Br. 225, salm. 75, Kl. 25, meat scrap 25.....	73	11.6	760	4,4	0.42	1.97	18.5	37	1.1
† Liver ext. 500, liver 50....	100	11.6	722	5,3	0.42	2.07	21.3	44	1.4
† Liver ext. 500, liver 50....	100	11.6	703	6,1	0.47	2.22	21.6	48	16.7
Br. 250, salm. 100, Kl. 25...	92	11.8	774	5,1	0.52	2.24	18.4	41	14.9
Br. 250, salm. 100, Kl. 25...	92	11.7	740	5,0	0.45	2.16	20.7	45	1.3
Dog 24–26. Bull, male, adult									
Br. 250, salm. 100, Kl. 25...	100	10.5	634	5,1	0.48	2.19	19.7	43	10.8
Br. 250, salm. 100, Kl. 25...	100	10.6	680	4,2	0.48	2.11	19.1	40	1.2
‡ Liver ext. 500, liver 100...	75	10.5	686	5,2	0.50	2.22	24.3	54	12.9
‡ Liver ext. 500, liver 100...	98	10.7	600	5,1	0.59	2.32	20.8	48	34.0
Br. 250, salm. 100, Kl. 25...	100	10.6	630	3,9	0.62	2.32	20.8	48	1.5
Br. 250, salm. 100, Kl. 25...	100	10.5	618	4,2	0.58	2.33	19.4	45	10.5
Br. 250, salm. 100, Kl. 25...	100	10.5	600	3,2	0.74	2.33	20.3	47	1.3

* Liver fraction supplemented by bread 200, salmon 75, Klim 25 daily.

† Liver fraction plus whole liver supplemented by bread 200 daily.

‡ Liver fraction plus whole liver supplemented by bread 250, Klim 25 daily.

whole liver. If we deduct the 13 gm. which would be due to the liver extract alone we have about 50 gm. hemoglobin per 2-week period as due to the 100 gm. of whole liver fed daily. This is more than would be expected and we may conclude that one fraction supplements the other and increases the potency of the combination.

The third experimental period shows the influence of the liver extract plus a liver ash. The preparation and potency of this ash have been reported recently (2). The ash fed daily represents 500 gm. whole fresh liver and when fed alone over a 2-week period in this type of experiment will be responsible for an increase in hemoglobin production of 40 to 50 gm. This particular dog (21–67) had not been tested for the liver ash effect but had been so tested for the apricot ash effect which gave 40 gm. hemoglobin production per 2-week period.

In this last experiment (Table 142) there is a considerable increase in blood hemoglobin as a result of the liver extract plus liver ash feeding. This amounts to about 68 gm. hemoglobin removed but we note the end hemoglobin level is 39 per cent or 7 points lower than the initial level of 46 per cent. This means the dog was bled about 8 gm. which are not to be credited to this diet period. We may credit to this liver extract plus the liver ash about 60 gm. hemoglobin per 2-week period over and above control periods. This is not far from the sum of the separate effects—15 gm. for the liver extract and 40 to 50 gm. hemoglobin for the liver ash. Also see Table 145 for lower values.

Table 143 gives more experimental data upon the effect of this liver extract supplemented by 50 to 100 gm. of whole liver. The liver extract, derived from 500 gm. of liver, fed daily shows about 15 gm. hemoglobin per 3-week period over and above the control periods—an average value. When we supplement the liver extract with 50 gm. of whole liver daily we observe an increase which is a little more than can be explained by the separate effects of these two factors. The hemoglobin actually removed is 28 gm. above the bread diet level of about 3 gm. hemoglobin per 2 weeks. The hemoglobin level at the start was 37 per cent and at the end 45 per cent which means that we should credit this diet period with 8 to 10 gm. hemoglobin more than was actually removed. A total effect of 35 gm. minus the liver extract effect of 15 gm. gives 20 gm. as due to liver feeding, 50 gm. daily. This value would scarcely be observed in feeding 50 gm. of liver daily for

2 weeks. Again we may conclude that the whole liver may increase somewhat the potency of the liver extract or *vice versa*.

The third experiment in Table 143 is upon another dog (24–26). The liver feeding is 100 gm. daily and the total hemoglobin increase due to 2-week liver feeding is 50 gm. above the bread diet level. This sum is larger than the liver extract value of 15 gm. hemoglobin plus the whole liver (100 gm.) feeding value as determined separately. The latter figure would average 15 to 20 gm. hemoglobin.

TABLE 144.

Liver Extract No. 343, N. N. R. Supplemented with Whole Liver.

Dog 25–97. Bull, male, adult.

Diet periods 1 wk. each	Food cons.	Wt.	Plasma vol.	RBC	Color index	Hb. index	RBC hemat.	Blood hb. level	Hb. removed bled
Food, *gm. per day*	%	*kg.*	*cc.*	*mil.*			%	%	*gm.*
Bread 500, salm. 100.....	96	17.2	1040	5,3	0.44	2.16	21.6	47	1.4
* Liver extract 500, liver 50.	100	17.0	968	6,0	0.46	2.14	21.1	45	18.1
* Liver extract 500, liver 50.	100	17.0	997	6,5	0.51	2.24	23.3	52	32.5
Bread 450, salm. 100.....	100	16.9	1008	5,4	0.47	2.21	23.2	51	13.8
Bread 450, salm. 100.....	100	16.6	1003	5,8	0.43	2.22	20.8	46	13.6
Bread 450, salm. 100.....	100	16.0	1100	5,3	0.44	2.16	21.6	47	1.4
† Liver extract 500.....	100	16.2	1099	5,8	0.40	2.08	22.2	46	1.4
† Liver extract 500.....	100	16.8	1116	6,3	0.43	2.04	21.2	43	16.4
Bread 500, salm. 100.....	100	16.8	1046	6,7	0.43	1.96	24.0	47	16.6
Bread 500, salm. 100.....	100	16.6	1054	5,6	0.44	2.00	24.3	49	12.7

* Liver fraction supplemented by bread 400 daily.

† Liver fraction supplemented by bread 500 daily.

Table 144 again gives experimental data in the same dog whereby we may compare the liver extract effect with the liver extract plus 50 gm. of whole liver daily. The values are all quite high and for the liver extract alone we observe an output for 2 weeks of 40 gm. hemoglobin above the usual base line. The liver extract combined with only 50 gm. whole liver daily gives the unusual total of 70 gm. hemoglobin output. Here again we note that this difference between 40

and 70 gm. hemoglobn is much more than could be expected as due to 50 gm. liver daily added to the basal bread ration.

We note further that this dog reacts with unusual hemoglobin production to all diets and meat products. There is an interesting fact which may be related to this uniformly high output in this dog. He was born and raised in our kennels. In the course of other experi-

TABLE 145.

Liver Extract No. 343, N. N. R. Fed with Liver Ash and Apricot Ash.

Dog 24–25. Bull, male, adult.

Diet periods 1 wk. each	Food cons.	Wt.	Plasma vol.	RBC	Color index	Hb. index	RBC hemat.	Blood hb. level	Hb. removed bled
Food, *gm. per day*	%	*kg.*	*cc.*	*mil.*			%	%	*gm.*
Br. 425, salm. 100.....	97	15.5	896	4,6	0.48	2.02	21.7	44	1.3
* Liver ext. 500, liver ash 500	96	15.6	888	5,6	0.47	2.08	27.8	58	16.0
* Liver ext. 500, liver ash 500	100	15.4	810	5,6	0.49	2.17	24.1	52	18.3
Br. 425, salm. 100.....	100	15.7	860	5,7	0.48	2.16	25.5	55	18.8
Br. 425, salm. 100.....	85	15.6	855	4,8	0.45	2.17	19.8	43	1.3
Br. 250, salm. 100, Kl. 25...	100	14.0	798	4,4	0.48	2.21	19.1	42	1.2
Br. 250, salm. 100, Kl. 25...	100	14.2	812	4,0	0.48	2.17	17.3	38	1.2
† Liver ext. 500, apricot ash 225.....	100	13.9	794	5,3	0.48	2.27	27.9	63	13.5
† Liver ext. 500, apricot ash 225.....	100	13.8	812	5,4	0.47	2.29	22.3	51	18.1
Br. 250, salm. 100, Kl. 25...	100	13.7	818	4,4	0.50	2.31	19.0	44	1.7
Br. 250, salm. 100, Kl. 25...	100	14.0	848	4,4	0.50	2.17	20.4	44	1.4

* Liver fraction and liver ash supplemented by bread 400, salmon 100 daily.

† Liver fraction and apricot ash supplemented by bread 250, salmon 100, Klim 25 daily.

mental work he was raised on a diet made up of at least one-half cooked liver and one-half standard salmon bread. He was on this diet for 29 weeks after weaning. When he was included in the anemia colony and the initial bleeding began it was found that he had an extraordinary hemoglobin reserve and always maintained an

unusually high level of hemoglobin production on various standard diets. This still continues even after 22 months of continuous severe anemia. Whether this is an individual peculiarity or a diet effect can be determined in time by using other dogs and will be of some interest if the diet is wholly responsible.

Table 145 gives more experimental data on liver and apricot ash to supplement the liver extract. These are to be compared with Table 142. The first experiment in Table 145 shows a hemoglobin production of about 50 gm. above the standard base line due to feeding the

TABLE 146.

Liver Extract No. 343, N. N. R. Supplemented with Fresh Dog Bile.

Dog 24–45. Bull, female, adult.

Diet periods 1 wk. each	Food cons.	Wt.	Plasma vol.	RBC	Color index	Hb. index	RBC hemat.	Blood hb. level	Hb. removed bled
Food, *gm. per day*	%	*kg.*	*cc.*	*mil.*			%	%	*gm.*
Br. 350, salm. 100.....	100	19.3	1132	4,7	0.47	2.19	20.3	44	1.4
* Liver ext. 500, bile 75.....	100	19.2	1038	4,9	0.48	2.17	21.4	47	1.4
* Liver ext. 500, bile 75.....	100	19.2	1079	5,0	0.46	2.24	20.5	46	1.4
Br. 350, salm. 100.....	100	19.0	1083	5,0	0.45	2.17	21.7	47	1.4
Br. 350, salm. 100.....	100	18.9	1145	4,8	0.52	2.14	21.4	46	15.2
Br. 350, salm. 100.....	100	18.8	1105	5,3	0.47	2.17	22.5	49	14.0
Br. 350, salm. 100.....	100	18.8	1087	4,6	0.49	2.21	20.5	45	1.3

* Liver fraction supplemented by bread 350, salmon 100 daily.

liver fraction plus the liver ash. We may account for 40 gm. hemoglobin by the liver ash alone, leaving only a little as due to the liver extract. In the second experiment we observe only 30 gm. hemoglobin production per 2-week period as due to the liver extract plus apricot ash feeding. All of this can be explained readily as due to the apricot ash intake.

Table 146 shows the negative effect of fresh bile added to the liver extract. The hemoglobin production above the standard base line due to bile plus the liver extract is delayed and amounts to about 25 gm. hemoglobin, all during the 2nd and 3rd weeks of "carry over."

There was no reaction during the 2 weeks of bile plus liver extract intake. We always hesitate to attribute a delayed reaction of this sort wholly to the preceding diet and believe that not more than half this hemoglobin is related to the liver extract plus bile. Bile feeding alone has no influence upon the hemoglobin production in this type of experiment (8). It would seem that bile does not supplement this liver extract any more effectively than it does the standard salmon bread basal ration.

Plasma volumes in these experiments show no noteworthy fluctuations. The record of these values gives much added security to the values of the circulating hemoglobin and therefore to the gross total hemoglobin production related to any diet.

DISCUSSION.

In another place (7) we have reported experiments with simple liver fractions and residues fed in this type of experimental anemia. We found that potent factors were present in the watery extract of liver, in the alcoholic extract and in the residue after watery and alcoholic extraction. In a general way one may say that our watery extract contains about one-fourth the potent material of whole liver, the alcoholic extract a little less than one-fourth and the residue a little more than one-fourth the potent material present in whole liver. About one-fourth was lost in the various chemical procedures.

These experiments lead us to the belief that several factors were present in whole liver which contributed to the production of new red cells and hemoglobin in this experimental anemia. It is not unlikely that during the stress of this sustained severe anemia the body is capable of breaking down and utilizing materials to manufacture red cells and hemoglobin which under normal conditions would be utilized for other purposes or thrown away as surplus waste.

It would seem probable that the red cell stroma matrix and the hemoglobin of the red cell would call for very different building stones coming from the food intake. We may assume that all reserve storage has been exhausted in these prolonged experimental anemias. We have made every effort to dissociate the hemoglobin production from the stroma production and still treasure the hope that this will some day be accomplished. Certain diets will modify the hemoglobin

index with considerable uniformity but there are many fluctuations which we cannot explain. We note in Tables 143 and 144 a sharp rise in the hemoglobin index associated with the liver extract plus whole liver feeding which does not appear with the liver extract alone. This suggests a more rapid hemoglobin production than stroma production giving a more complete saturation of the stroma with the blood hemoglobin. We can control the hemoglobin production with much more certainty than can be done with the stroma production. Apparently the body can manufacture stroma more readily from these various diets than is the case with hemoglobin. We had hopes that this liver extract No. 343, N.N.R. might cause some dissociation of the stroma-hemoglobin ratio but we can record no evidence for this—note figures for the hemoglobin index in various tables.

CONCLUSIONS.

A liver extract No. 343, N.N.R. known to be fully potent in pernicious anemia shows but 10 to 20 per cent of the potency of whole liver feeding in severe continuous secondary anemia due to bleeding in dogs. There are wide individual variations which are not noted with whole liver feeding.

Supplementing this liver extract with whole bile does not modify the reaction.

Supplementing this liver extract with liver ash or apricot ash shows the sum of the two expected feeding reactions.

When we supplement this liver extract with small amounts of whole liver (50 to 100 gm.) we may observe hemoglobin and red cell production in excess of the sum of the expected separate reactions. Whether the liver extract increases the potency of the whole liver feeding or *vice versa*, it suggests similar possibilities in various human secondary anemias.

Liver and kidney fractions of varied types deserve much study to ascertain their effect upon widely divergent types of human anemias.

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STUDIES ON THE SUBMAXILLARY VIRUS OF GUINEA PIGS.

I. The Effect of Duct Ligation and Pilocarpine Administration upon the Cellular Response to the Virus.

By GORDON H. SCOTT, Ph.D.

(From the Laboratories of The Rockefeller Institute for Medical Research, New York, and the Department of Anatomy, Washington University School of Medicine, St. Louis.)

Plates 18 to 20.

(Received for publication, October 6, 1928.)

Little is known of the exact relationship existing between any of the filtrable viruses and the cells injured by their action. An obvious line of investigation is to alter the functional activity of the cells and to note how this modifies them in respect to the viruses. The purpose of the present paper is briefly to report some experiments in this direction. The submaxillary virus of the guinea pig was selected, in preference to other filtrable viruses, mainly for three reasons: first, because it can be easily obtained from the majority of old guinea pigs; second, because the nuclear inclusions produced are larger and more conspicuous than those called forth by any other known virus; and lastly for the reason that the activity of the submaxillary gland may readily be influenced by many experimental procedures.

Duct Ligation.

In the first place it was decided to modify specifically and radically the cells attacked by the virus, through ligation of the submaxillary duct. This was accomplished in the following way:

With the animal under ether anesthesia a median longitudinal incision was made in the skin of the neck. The superficial muscles and fascia were pushed aside with blunt instruments and the duct located and grasped with forceps. A ligature of white silk thread No. 5 was then passed around the duct and tied securely, care being taken to avoid injury to the vascular and lymphatic supply of the gland. Finally the incision in the skin was closed with heavy silk thread and the surround-

ing area was painted with iodine. In every case healing occurred without infection.

For the experiments 32 guinea pigs, 3 weeks old and approximately of the same weight, were divided into 4 lots, and a fifth lot of animals unoperated upon, of the same age was added to the series for control.

Lot 1 consisted of 16 animals in all of which the duct of the right submaxillary gland was ligated. The first injection of virus was made subcutaneously 12 hours after ligation. This was followed by two other injections spaced 24 hours apart.

The virus was freshly prepared in each instance by taking the submaxillary glands of an adult guinea pig and grinding them in a sterile mortar. Enough salt solution was added to make about a 10 per cent emulsion of gland substance. This was given in 0.5 cc. doses. Small pieces of the same tissue fixed in Zenker's fluid and colored with Giemsa's stain, when examined, revealed the presence of typical nuclear inclusions in each gland used in the preparation of virus.

Lot 2 was made up of 4 guinea pigs in all of which the right submaxillary duct was ligated but which received no virus. The animals were killed 9, 12, 15 and 18 days after the operation. This was therefore a control group.

Lot 3 consisted of 4 animals which had both right and left submaxillary ducts ligated and which were given virus like the members of Lot 1.

Lot 4, again 4 guinea pigs, was a control on Lot 3, the ducts on both sides being ligated but no virus being given.

Lot 5 consisted of 4 animals unoperated upon which received virus as did those in Lot 1.

Reaction of Unligated Glands to the Virus.

The alterations will be compared; first, with those found in typical induced infections (Lot 5), and, second, with others occurring spontaneously.

With but one exception the infection in these unligated glands in Lot 1 was much more extensive than that in animals which had not been operated upon yet received a similar dosage of virus (Lot 5). Perhaps this was because the amount of normal gland substance offered to the virus was reduced by one-half owing to the ligation of the ducts on the right side. But it may have been a question of rate of physiological activity rather than the quantitative one of availability of gland substance for the unligated glands in Lot 1 were obliged to care for the duties normally performed by their ligated mates.

An attempt was made to represent this difference in a roughly quantitative way by counting the number of inclusion-containing nuclei as compared with the visibly unaltered nuclei present. In the

estimation every duct with inclusions seen in a median cross-section of the gland was considered; but the nuclei were not counted in cross-sections of ducts devoid of inclusions. A transverse section of one of the glands of Lot 1 contained 56 sections of ducts exhibiting inclusions, but such a very heavy infection was unusual.

The counts gave a ratio for the unligated glands (Lot 1) of nuclei with inclusions to unaltered nuclei varying from 1:3.42 to 1:5.59; while the proportion of normal nuclei was much higher in the controls (Lot 5), namely from 1:4.79 to 1:8.60. It is to be noted further that the number of ducts affected was also less.

Not only was there this difference between the relative number of inclusions in the animals of Lots 1 and 5, but also a difference was noted in their comparative distribution. They were found in a wider variety of cells. Instead of being confined to the cells of the secretory ducts they were observed quite frequently in the acinous cells, the cells of the intercalated ducts and even appeared occasionally in those of the collecting ducts.

Likewise in the mucous portion of the left unligated glands inclusions were found in some cases in the acinous cells in addition to the secretory ducts in which latter they were not seen in the 4 animals of Lot 5.

The second comparison with spontaneous infections was more worth while, because it was made on a more adequate scale—16 animals in Lot 1 and 42 adults with spontaneous lesions. Here the same two differences were noted. The inclusions were both more abundant and were spread in a larger variety of cells in the unligated left glands (Lot 1) than in the spontaneous lesions of old animals.

The detailed appearance of inclusions in spontaneous lesions in animals less than 1 month old has apparently not been recorded. Their occurrence in guinea pigs so young, which alone would be comparable as to age with the animals of Lot 1, is negligible. Cole and Kuttner (1) found only 3 such cases among 43 animals less than 1 month old and they made no reference to the existence of any difference in the distribution of the inclusions. Spontaneous infections were never observed in any of the guinea pigs about 3 weeks old studied in connection with these experiments.

Reaction to the Virus of the Ligated Submaxillary Gland.

It may be said at once that ligation of the right submaxillary ducts in the guinea pigs of Lot 1, and of the ducts on both sides in Lot 3, completely inhibited the formation of any intranuclear inclusions within the glands, whereas, as has been stated, the unligated glands on the left side (Lot 1) developed many inclusions.

The appearance of the epithelium after duct ligation is illustrated in Figs. 2, 4 and 6. Changes of several kinds were recognized. These seemed to be the same in control ligated animals (Lots 2 and 4) which did not receive any virus.

The epithelium of the ducts proliferated extensively (Fig. 2). The division was by karyokinesis and looked regular. No multipolar mitoses were detected, nor signs of amitosis. A characteristic metaphase is illustrated in Fig. 2. The prophase represented in Fig. 4 looks in the photomicrograph something like an inclusion but in the preparation it could be distinguished clearly from the typical inclusions shown in the figure above it of the corresponding unligated left submaxillary gland. The proliferation was limited by the connective tissue surrounding the ducts and separating them from the acinous tissue. As a result of the growth of cells there was considerable vacuolation and pressure on the cells, particularly those lining the lumen, into which cavity they frequently desquamated (Fig. 2). The multiplying cells possessed mitochondria which were normal in shape and in number.

The cells of the secreting acini, on the contrary, did not multiply but underwent extensive degeneration. Sections from glands 2 to 5 days after ligation of their ducts showed merely a depression in the affinity of the acinous cells for Giemsa's stain and a change from rod-like to small spherical mitochondria. But 9 days after ligation very little recognizable acinous tissue remained, its place having been taken by connective tissue and by clumps of mononuclear cells. At no time, however, were lymphocytes or polymorphonuclear leucocytes abundant. One of the latter is to be seen within the proliferating duct epithelium in the upper part of Fig. 2.

The absence of intranuclear inclusions in the ligated glands suggested the possibility that the virus might also be absent. To secure information on this point the following experiment was made:

5 young guinea pigs were selected and the right submaxillary duct was ligated in each animal. Three inoculations of fresh virus, prepared as in the manner described, were given at 24 hour intervals. 16 days after the last inoculation the animals were killed and an emulsion prepared from their combined ligated glands. The unligated glands of this series were examined in the usual manner and all of them showed typical virus infections with many intranuclear inclusions in the secretory ducts. The virus thus secured was injected by the intracerebral route into 5 young guinea pigs each of which received a dose of 0.1 cc. They showed no untoward symptoms after their recovery from the ether anesthesia and were allowed to live for 4 weeks when their brains were removed and examined for inclusions.

In one of the five brains three cells were located whose nuclei contained intranuclear bodies only slightly resembling typical inclusions; but there was little or no meningeal exudate. The remainder of the brains were uniformly negative although a diligent search was made for inclusions.

It seems probable therefore that the virus was absent in the ligated glands in this series or else was present in such small amounts, or so attenuated, as to be incapable of manifesting itself in the usual manner. But there is still another possibility that the animals inoculated intracerebrally were possessed of an active immunity against the virus (2). This is, however, extremely unlikely because, as has been noted, spontaneous infections are rare in guinea pigs less than 1 month old. This observation, that the virus is in all likelihood absent in ligated glands, is interesting in view of the fact that every precaution was taken not to interfere with the blood and lymph supply of the gland in the process of ligation. In sections of the ligated glands blood and lymphatic vessels that were apparently normal could be identified with ease. The conclusion seems justified that through this special sort of injury the virus is warded off.

The Effect of Pilocarpine Administration.

The severity of the infections obtained in the unligated glands (Lot 1), which were working overtime to compensate for the throwing out of commission of the ligated glands on the opposite side, rather pointed to the conclusion that heightened physiological activity favored a severe reaction to the virus. To test this possibility another experiment was performed.

6 young guinea pigs of about the same weight were chosen. All of them received three inoculations of fresh virus at 24 hour intervals. In addition, 5 of the guinea

pigs were given doses of pilocarpine hydrochloride of sufficient amount to produce a considerable degree of salivation. The dosage varied with the weight of the animal and with its individual idiosyncrasies in respect to the drug. The first 2 days of the experiment were sufficient to establish the dosage which would produce an extensive flow of saliva but not kill. Increasing amounts of pilocarpine were given as the experiment progressed for the animals acquired some tolerance. An almost continuous flow of saliva was finally produced in each guinea pig for at least 12 hours out of each day for 15 days. One animal of the 6 was reserved for a control and was given an equivalent amount of physiological salt solution subcutaneously each day in place of the pilocarpine.

At the end of 15 days the animals were killed and their salivary glands prepared for examination by the procedure already outlined.

The submaxillary glands of the control animal presented the usual nuclear inclusions in the cells of the secretory ducts. The severity of the infection was not marked. For example, in one section taken through the center of the gland only 50 inclusions could be counted.

The glands from the members of this series which had been stimulated with pilocarpine, showed by contrast a complete exhaustion of the acinous cells. In some of the glands, on the other hand, there was evidence of hyperplasia in the acinous tissue. Mitotic figures were occasionally found in such regions, and it was quite evident that newly formed secretory tissue was present. There was considerable edema of the interstitial tissue.

Nuclear inclusions were present in large numbers. That the infection was active at the time the animal was killed is demonstrated by the preponderance in number of "young" inclusions visible in the treated glands. Literally every section of a secretory duct was the carrier of one or more inclusion-laden nuclei. Often as many as 20 altered nuclei were present in a single section of a duct. In one instance a section through the center of a treated gland presented 312 typical inclusions. This proved to be an unusually large count as the average of sections counted was 203. It is evident, therefore, that pilocarpine stimulation did lead in some way to the development of a markedly increased number of inclusions in this series of animals.

But this is not all, for again was encountered the peculiar involvement, found in the ligation experiments, of cells which usually escape virus action, namely the acinous cells and the cells of the intercalated and collecting ducts. Compensatory overactivity and heightened

activity caused by pilocarpine therefore clearly alter the relationship between virus and cells in much the same way.

DISCUSSION.

The experiments described show that the incidence of intranuclear inclusions (and quite possibly of virus) may be altered at will by experimentally modifying the physiological activity of the salivary glands on which the submaxillary virus acts.

The formation of inclusions may be completely inhibited by ligation of the submaxillary duct. This result came as a surprise because the operation causes injury and the active formation of young cells—two factors which are believed by Rivers (3) and others to be influential in the promotion of virus action.

Conversely, the development of inclusions may be greatly aided by stimulation of the gland with pilocarpine. There is often in such instances an active formation of young cells, but it is not accompanied by injury other than that resulting from a high degree of functional activity maintained to a stage of profound exhaustion.

The differential factor in the response is not the presence or absence of young cells, but rather the depressed or heightened functional activity artificially imposed upon the glands at the time that they are subjected to virus action.

SUMMARY.

The development in the submaxillary glands of guinea pigs of intranuclear inclusions traceable to submaxillary virus can be wholly suppressed by ligation of the gland ducts. Inclusions appear in far greater numbers in glands stimulated with pilocarpine than is the case in ordinary spontaneous and induced infections.

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EXPLANATION OF PLATES.

All the photomicrographs were taken of preparations fixed in Zenker's fluid and colored with Giemsa's stain at a magnification of 1500 diameters. The upper figure in each case represents a portion of the left, unligated, gland and the lower figure one from its mate, the ligated, right, submaxillary gland of the same animal.

PLATE 18.

FIG. 1. Inclusion-laden cells in a guinea pig of Lot 1 killed 9 days after the last virus inoculation.

FIG. 2. Ligated gland from the same animal showing proliferation of duct cells (note metaphase about the center of the figure), and their desquamation into the constricted lumen; but no inclusions.

PLATE 19.

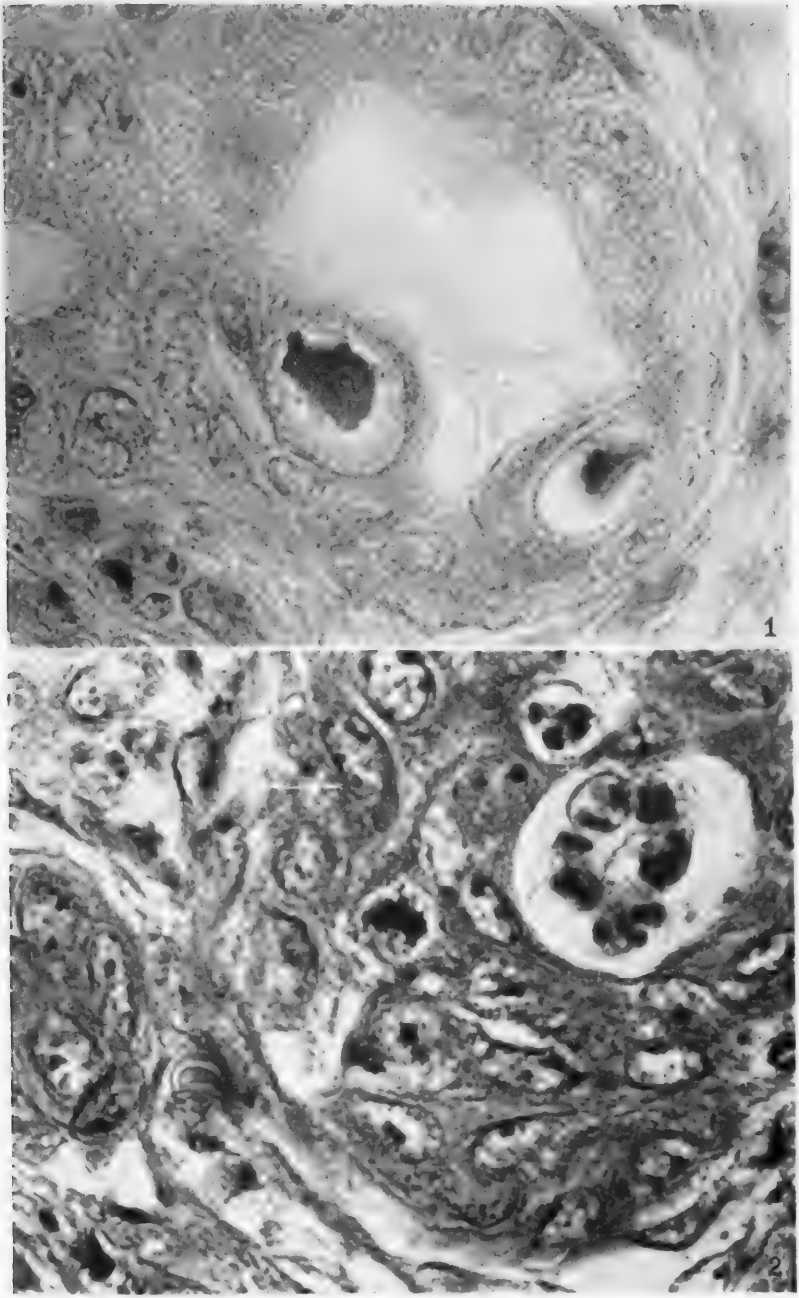
FIG. 3. Cells possessed of nuclear inclusions in a guinea pig of Lot 1 killed 11 days after the final inoculation with virus.

FIG. 4. Ligated gland from same animal illustrating active multiplication of cells and still further occlusion of the duct. The cell to the left of the duct, which is near the center of the figure, shows a division prophase.

PLATE 20.

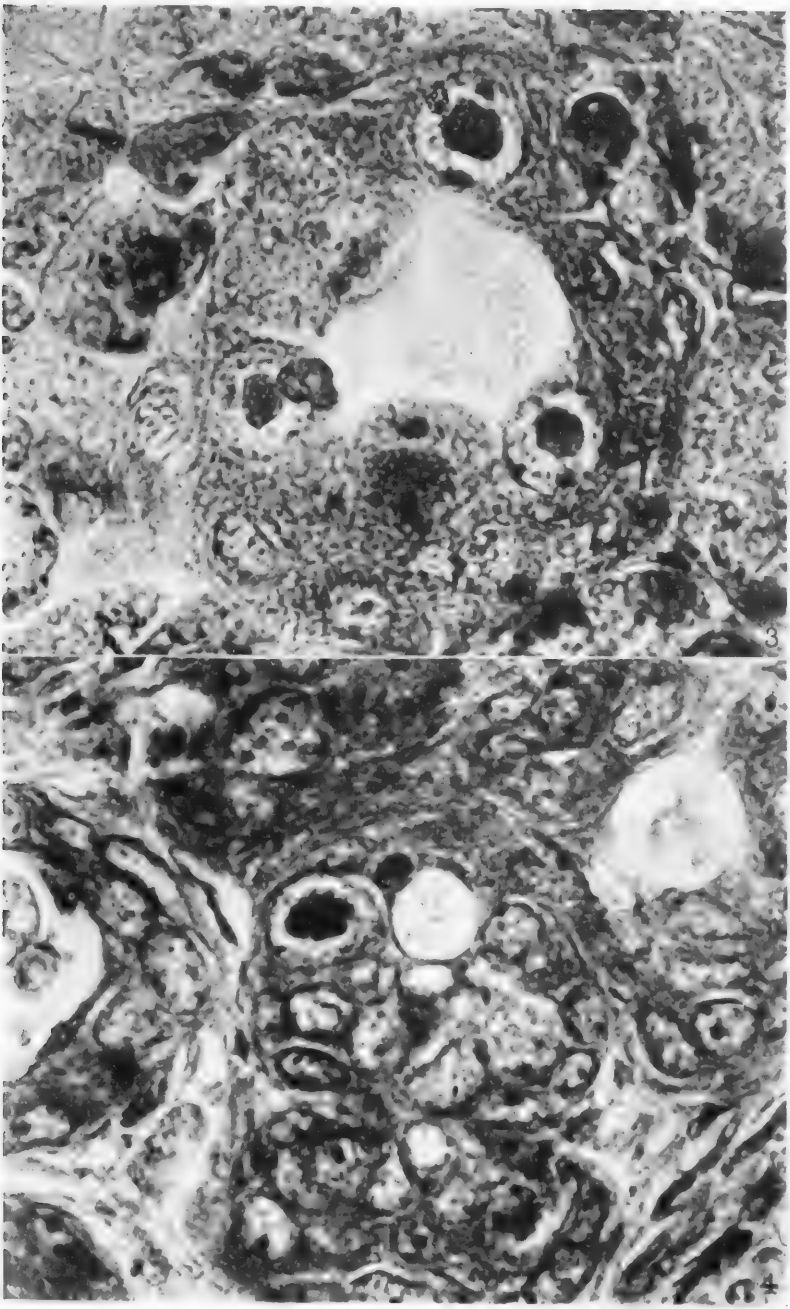
FIG. 5. Cells with inclusions in a guinea pig of Lot 1 killed 13 days after the last injection of virus. The dark tint of the cells in this figure as compared with Fig. 1 is due to a difference in photographic exposure, not to a variation in intensity of staining.

FIG. 6. Ligated gland from the same animal. A metaphase is conspicuous in the epithelium of the duct which at this point consists of a single layer of cells. There are no inclusion bodies.



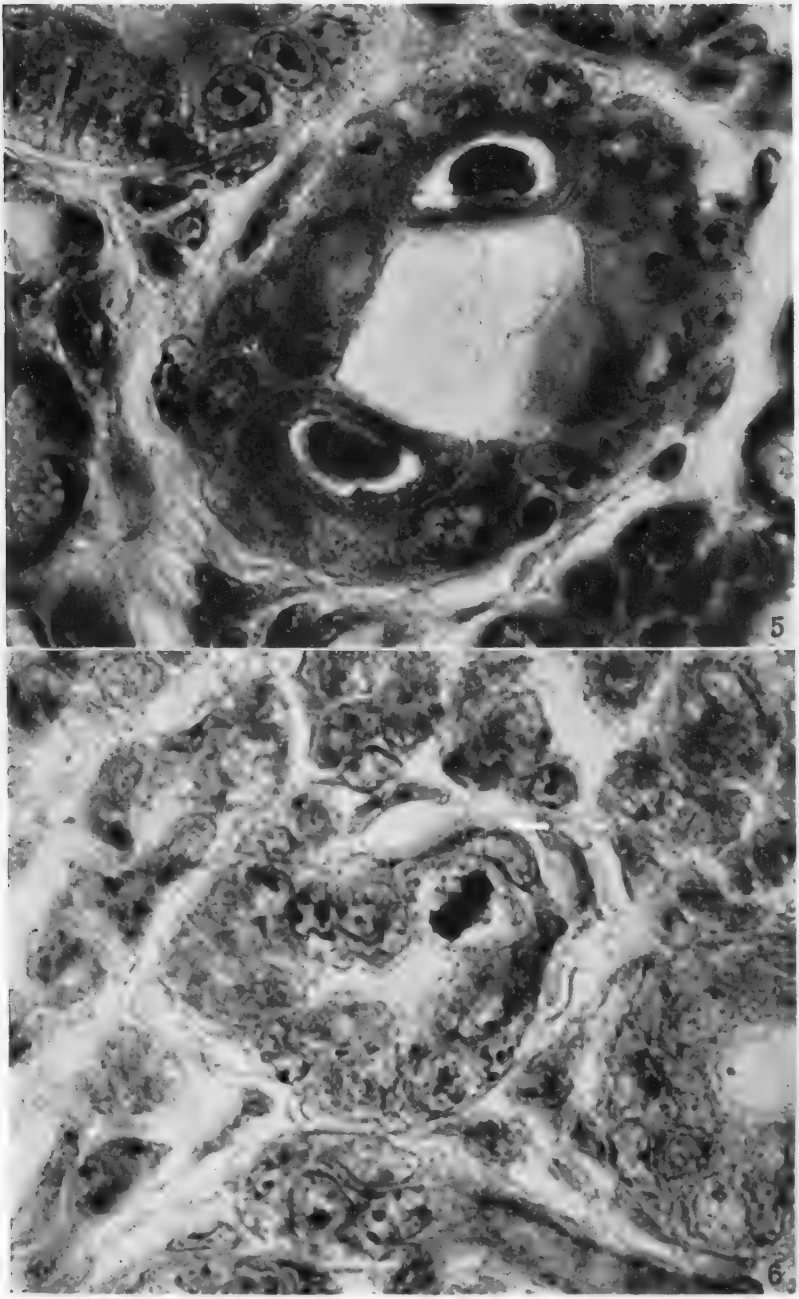
Photographed by Louis Schmidt.

(Scott: Submaxillary virus of guinea pigs. I.)



Photographed by Louis Schmidt.

(Scott: Submaxillary virus of guinea pigs. I.)



Photographed by Louis Schmidt.

(Scott: Submaxillary virus of guinea pigs. I.)

THE REVERSION OF R TO S PNEUMOCOCCI.

By HOBART A. REIMANN, M.D.

(*From the Department of Medicine, Peking Union Medical College, Peking, China.*)

(Received for publication, October 8, 1928.)

Extensive studies on microbic dissociation have led to much speculation regarding the significance of this phenomenon to infectious disease. As an outcome of many studies on the dissociation of Pneumococcus, there has been a tendency to imply that R pneumococci ordinarily appear during the course of pneumonia as a result of the dissociation or degradation of the pathogenic type-specific S forms. It was suggested that in turn many of the R forms under favorable circumstances acquired the characteristics of non-pathogenic Group IV strains which are so frequently encountered in the sputum of both healthy individuals and patients suffering from pneumonia or convalescent from this disease. It was assumed that these bacteria residing in the sputum as pure saprophytes await conditions favorable to a reversion either directly or indirectly to pathogenic S forms and thus acquire the capacity to invade the host again, completing the cycle.

This view does not seem altogether unreasonable in view of the fact that typical R forms can be induced experimentally *in vivo* (1) and that R forms occur, though uncommonly, in the sputum of patients suffering from pneumonia (2). Other investigations (3, 4) have shown that R forms may revert completely to the original virulence and specificity of the type from which they were derived. Recently Griffith (5) has shown experimentally that by special treatment *in vivo* certain R forms may be made to assume the characteristics of either Type I, II, III, or Group IV pneumococci regardless of the type from which the R forms were originally derived.

The present paper contains a report of further studies on the behavior of certain R strains under various experimental conditions with reference to their relationship with Group IV pneumococci. An investigation of the transformation of type as reported by Griffith was also made.

EXPERIMENTAL.

Experiments with Type I Pneumococci.

A strain of Type I-S pneumococcus was transferred repeatedly in broth containing 10 per cent Type I antipneumococcus serum in the manner commonly employed to induce the appearance of R forms. Agar plates made from each passage were examined under the lowest power objective of the microscope by reflected daylight from the surface of the tilted plate. After the 6th transfer many R colonies appeared among the S forms. When the R colonies were examined closely after 48 hours incubation the rough surfaces of many of them were studded with small shiny hummocks or papillæ. This phenomenon has been observed among S colonies in previous investigations and has been described by Paul (6). A shiny portion designated as a daughter or secondary colony and a portion of the rough base were touched with a fine needle and plated separately for further examination and will be described later.

The passages in 10 per cent Type I serum broth were continued until 42 transfers had been made. After every other transfer blood agar plates were made and examined and 1 cc. of the culture was inoculated intraperitoneally into white mice. After the 8th transfer no more of the typical shiny S colonies were seen on the blood agar plates and the organisms thereafter were generally avirulent for mice. However, mice inoculated with bacteria of the 11th, 34th, and 35th transfers died and typical I-S organisms were recovered from the blood. It appeared that some of the bacteria were occasionally able to revert to the original virulent form, even after prolonged treatment in specific immune serum.

Plates made from the successive transfers showed a considerable variation in the morphology of the R colonies. At times the colonies were large and thick and again small and thin. Occasionally mixtures appeared but both had rough surfaces. Daughter forms or shiny papillæ on the R colonies appeared occasionally.

After the 15th transfer and during the subsequent transfers a new form of colony occasionally appeared in varying proportions to the R forms and R forms with daughter colonies. The new colony was usually larger, thicker, and more succulent than the typical R form, and save for the very finely granular appearance of its shiny surface was morphologically indistinguishable from the original Type I-S colony. Bacteria from the new form of colony, however, were avirulent for mice and were serologically similar to R pneumococci. Apparently the new forms represented isolated daughter colonies.

Experiments with Daughter Colonies.

Attempts were then made to determine the significance of the daughter or secondary colonies. A study was made of the biological characteristics of the organisms comprising the rough base of the R

colony and of those derived from the daughter colony after the 6th passage in immune serum broth.

(*a*) *Colony Morphology.*—Bacteria taken separately from each form were repeatedly transferred on blood agar and observed throughout 18 transfers. For each transfer of the bacteria composing the rough base, care was taken to select organisms from only the rough portion without admixture of the daughter colony forms. Cultures on blood agar of the daughter strain showed the presence of homogeneous, shiny, convex colonies alone. Cultures of the bacteria picked from the rough base each time revealed a mixture of varying proportions of plain rough colonies, rough colonies with daughter papillæ, and separate shiny daughter colonies. The tendency for the relative proportion of the separate daughter colonies to increase was noted as the transfers succeeded.

It would appear then that under these conditions the daughter colonies represent a more stable variety of the attenuated forms of pneumococci.

The individual cocci of the daughter colony when stained did not show capsules and usually appeared to be considerably smaller than organisms of either the typical R or S forms. No capsule was demonstrable by the India ink method. Organisms from the R colony on the other hand varied considerably in size and often showed bizarre forms.

(*b*) *Agglutination Tests.*—The agglutination reaction tested by employing the usual thread test was applied to 5 strains isolated from characteristic daughter colonies which were selected at different times during the transfer experiment. A typical R strain and an S strain were used as controls. Since the serological reactions of all 5 daughter colonies were identical, the reaction of only 1 is illustrated in Table I.

It is evident from the results shown in the table that the bacteria from daughter colonies and those from the typical R colony are serologically alike, in spite of the striking morphological differences of the colonies.

(*c*) *Virulence Tests.*—Many virulence tests were applied to various daughter and R colony cultures by the usual method of inoculating mice intraperitoneally with 1 cc. of the culture. Although mice occasionally succumbed to this procedure there was no indication of a reversion to the S form of any of the strains. Cultures made from the blood of the mice which died were avirulent and non-specific.

On one occasion, however, 2 rabbits, and 2 guinea pigs were inoculated intraperitoneally with the growth, 5 cc., of broth cultures of 2 daughter colonies selected from R colonies after the 15th transfer. Both of the rabbits and 1 of the guinea pigs died within 48 hours and Type I-S pneumococci were recovered from the blood. Control animals inoculated with 2 strains of typical R forms survived.

From these experiments it appears that although R and daughter strains produce colonies which are not morphologically alike, they are serologically similar and are usually avirulent for mice. On one

TABLE I.

Comparative Agglutination Reactions of I-S, I-R, Daughter, and AF Pneumococcus Strains.

Serum dilutions	I-S strain				I-R strain				Daughter strain				AF strain			
	Antipneumococcus serum			Normal horse serum	Antipneumococcus serum			Normal horse serum	Antipneumococcus serum			Normal horse serum	Antipneumococcus serum			Normal horse serum
	I	II	III		I	II	III		I	II	III		I	II	III	
1:10	Disc	−	−	−	+++	+++	+++	+	+++	+++	+++	+	+++	+++	+++	+
1:100	Disc	−	−	−	+++	++	++	−	+++	++	++	−	++	++	++	−
1:1000	−	−	−	−	++	+	+	−	++	++	+	−	++	+	+	−
1:10,000	−	−	−	−	+	−	−	−	−	−	−	−	+	+	−	−

+++ coarse clumping.
++ clumping.
+ fine clumping.
− diffuse.

occasion 2 daughter colonies reverted to the S form after inoculation into rabbits and guinea pigs.

Experiments with Agar Foci Containing R Pneumococci.

In a previous study (1) it was reported that a strain of I-R pneumococci survived for 5 weeks in an agar focus embedded subcutaneously in a guinea pig. By using the same technic it seemed desirable to observe in more detail the effects on an R strain of prolonged exposure to living body influences.

Agar foci inoculated with I-R organisms and others inoculated with daughter colony organisms were established subcutaneously in 8 mice according to the technic given. Observations were made at 3 to 7 day intervals by aspirating some of the material from the foci with a hypodermic needle and syringe and plating it on blood agar plates.

Long residence *in vivo* produced effects on both R and daughter strains similar to those observed after repeated transfer in specific immune serum broth or upon blood agar plates mentioned in the previous experiments. Repeated examinations of plates made from the foci inoculated with the daughter strain over a period of 44 days revealed the presence of shiny colonies alone. On the other hand the usual variety of rough colonies, rough colonies with daughter papillæ, and daughter forms alone appeared in varying proportions upon plates made from the foci originally inoculated with the R strain. Several of the mice died during the experiment from other causes. Reversion to the S form never occurred.

A shiny colony strain, designated for convenience as Strain AF, was isolated from an agar focus on the 34th day after inoculation, and was used for further study in experiments to be reported later. This strain thus far has never reverted to the S form and according to Table I is serologically similar to the R and daughter strains.

(*a*) *Virulence and Immunizing Properties of Strain AF.*—Since on a previous occasion 2 daughter strains reverted to the S form after inoculation into rabbits and guinea pigs, it was desired to ascertain the effect of attempts to reduce the resistance of mice on the capacity of the AF strain to revert.

For this purpose 5 mice in which agar foci inoculated with the AF strain had been established subcutaneously, were intoxicated by the intraperitoneal injection

of 1 cc. of a 10 per cent solution of alcohol. Following this the mice were frequently wetted with cold water and alternately placed at half-hourly intervals in the ice box at 3°C. and in the incubator at 37°C. for a period of about 12 hours. During this experiment 1 of the mice died and on several occasions the others appeared to be moribund but revived when warmed in the incubator. In spite of this treatment none of the remaining mice succumbed to pneumococcus infection.

A single experiment was made to determine whether or not mice harboring the AF strain in agar foci were immune to small doses of I-S pneumococci.

3 normal mice and 3 mice in which the AF strain had been present in agar foci for 7 days, were inoculated intraperitoneally with .0001 cc. of a culture of I-S pneumococci. All of the mice died approximately 72 hours later. Apparently the AF or daughter strain conferred no immunity against I-S pneumococci.

Experiments with Strains Isolated from Sputum Cultures.

In an attempt to detect R colonies in sputum cultures Paul (2) was able to isolate only 2 typical R strains from the 40 rough surfaced methemoglobin-producing colonies which he selected. As a result of the present study it is evident that the rough surface of colonies cannot always be depended upon as a guide to the recognition of the dissociated forms of pneumococci. With the fact in mind that "R" colonies may have shiny surfaces, similar efforts were again made to detect R strains in sputum cultures. Cultures on blood agar plates were made from the sputa of 4 lobar pneumonia patients shortly after the crisis following Type I infection. Colonies surrounded by a greenish hue and having a shiny or finely granular surface after 48 hours incubation were selected. Cultures from 72 colonies were tested. Of these 30 were discarded as bile-insoluble, 9 proved to be I-S strains, and the remainder which ordinarily would be regarded as Group IV strains were studied further.

Upon replating the last group on blood agar plates most of the colonies did not resemble either the typical R or daughter colonies, the serological reactions of the organisms were not characteristic, and many of the strains showed slight but constant virulence for mice. No typical R strains were encountered.

It is evident therefore that although R strains of pneumococci have been isolated from the sputum, they are by no means common.

Experiments on the Transformation of Type.

Although the transformation of pneumococci of one type into those of another type has been reported on several occasions in the past, convincing evidence that this change may occur did not appear until Griffith's publication (5). His results were confirmed by Neufeld and Levinthal (7). The method by which this change was induced consisted in inoculating a small quantity of living R organisms, together with the deposit of a large amount of heat-killed S culture, subcutaneously in mice. The R strain appeared to be converted with considerable regularity into either the type corresponding to the heat-killed S type used or to its own original type depending upon the characteristics of the strain. Different R strains varied in their ability to undergo this change. Griffith showed that the apparent transformation never occurred directly from one type into another but always through a process of evolution by passing through the R or dissociated stage.

Griffith's methods were applied to a number of R strains, the AF strain, and to strains derived from the sputum of convalescent pneumonia patients. The results are given below.

Methods.

Strains of Type I, II, or III pneumococci were grown in 1 per cent dextrose broth for 5 to 6 hours until a dense growth occurred. The tubes containing the cultures were then sealed and completely immersed in a water bath at 60°C. for 40 minutes following which the cultures were centrifuged. Mice were inoculated subcutaneously in the inguinal region with doses representing the deposit of from 10 to 30 cc. of the heated S culture together with .25 cc. of a living R culture. Many control tests were made to determine the possible survival of some of the S forms in the heated cultures. Viable S forms were never demonstrated in any culture heated to 60°C. longer than 15 minutes.

The R cultures used were derived in the usual manner by cultivating S strains in homologous immune serum broth. 2 R strains, the AF strain from the agar focus, and 19 strains of pneumococci isolated from the sputum of convalescent pneumonia patients were employed. Attempts were made beforehand to induce reversion of the R and AF strains to the S form by using methods which have previously been found suitable to induce the R→ S change. The cultures were repeatedly grown in anti-R serum broth, repeatedly passed through mice, and inoculated in massive doses (deposits of 25 to 100 cc.) subcutaneously in mice without success. The strains appeared to be irreversible.

TABLE II.

Transformation of the Strain AF (Originally Derived from I-S) to S Strains.

Living R form inoculated	Heat-killed S form inoculated	Mouse No.	Result	Result of agglutination test of the heart's blood culture					
				Antipneumococcus sera					
				Type I		Type II		Type III	
				1:1	1:10	1:1	1:10	1:1	1:10
.25 cc. AF strain	Deposit of 25 cc. Type I-S culture	1	Died 48 hrs.	++++	+	−	−	−	−
		2	Died 96 hrs.	++++	+	−	−	−	−
		3	Died 72 hrs.	+++	+	−	−	−	−
		4	Died 20 hrs.	++++	+	−	−	−	−
		5	Died 72 hrs.	++	++++	−	−	−	−
		6	Died 78 hrs.	++	++++	−	−	−	−
		7	Survived 7 days						
		8	Survived 7 days						
		9	Survived 10 days						
		10	Survived 10 days						
		11	Died 6 days		Contaminated				
		12	Survived 7 days						
		13	Survived 7 days						
		14	Survived 7 days						
.25 cc. AF strain	Deposit of 25 cc. Type II-S culture	1	Died 18 hrs.	++	++	++	++	++	++
		2	Survived 10 days						
		3	Survived 10 days						
		4	Survived 10 days						
		5	Died 72 hrs.		Sterile				
		6	Died 7 days	++++	++	−	−	−	−
		7	Survived 7 days						
		8	Survived 7 days						

.25 cc. AF strain	Deposit of 25 cc. Type III-S culture	1	Died 72 hrs.	−	−	−	+	++
		2	Died 96 hrs.	−	−	−	+++	++
		3	Survived 10 days					
		4	Survived 10 days		−	−	++	++++
		5	Died 120 hrs.	−	−	−	+++	++++
		6	Died 144 hrs.	−	−			
		7	Survived 10 days					
		8	Survived 10 days					

Experimental.

Strain AF which had resided for 34 days *in vivo* in an agar focus was first employed. It will be recalled that this strain was derived from a I-R strain which in turn originated from a I-S culture. Strain AF was inoculated together with heat-killed cultures of Types I, II, and III pneumococci. After a few days a number of the mice died and cultures from the blood revealed the presence of type-specific pneumococci. Results of a typical experiment are illustrated in Table II.

Apparently, according to the table, Strain AF in many instances reverted to the S form of Type I or transformed into Type III according to the heated culture used. Transformation into Type II did not occur although it appears that in one case Type II-S heated culture induced a reversion of the AF strain to its I-S prototype.

In a similar manner typical R forms derived from Types I and II pneumococci were then inoculated with heated cultures of Types I-S, II-S, and III-S. Similar results were obtained. I-R organisms inoculated with heated II-S culture appeared to be transformed into II-S organisms. II-R pneumococci were changed into either Type I-S or Type III-S forms depending upon the type of the heated culture used. Both I-R and II-R strains frequently reverted into their respective prototypes I-S and II-S when inoculated together with the heat-killed cultures of their homologous type S forms.

Experiments with Pneumococci from Sputum Cultures.—It was then considered to be of importance to apply this technic to some of the pneumococcus strains which were isolated from the sputum cultures, since it has been suggested that such strains may be potential S forms. Accordingly 19 of the pneumococcus strains were selected and inoculated into 2 series of mice. In 1 series the strains were inoculated together with the deposit of 10 cc. of heat-killed I-S culture, the other with the same amount of heat-killed II-S culture. Of the 97 mice inoculated, 59 survived, and the rest died of Pneumococcus Group IV septicemia. No reversion or transformation to either Type I-S or II-S occurred. In considering this result however, it must be borne in mind that the sputum strains were not typical R forms and therefore do not conform to Griffith's contention that transformation can only occur through the R stage.

Experiments with Heat-Killed Friedländer Bacilli.—Since Griffith has emphasized the importance of the "S antigen" as a stimulus to the transformation of type, further experiments were made by substituting Type II-S heat-killed pneumococci with Strain E Friedländer bacilli.

According to Avery and Heidelberger (8) a striking chemical and immunological similarity of the capsular substances (specific soluble substance) exists between two special types of these two widely different forms of bacteria. It was desired therefore to test the ability of the heated culture of Friedländer bacilli to induce transformation of R bacteria to Type II-S pneumococci.

An E strain of Friedländer bacilli agglutinating specifically in Type II-S antipneumococcus serum was grown, heated, and centrifuged in the usual manner. The deposit of 20 cc. of heated culture was inoculated with living cultures of I-R, II-R, and Strain AF pneumococci. Neither transformation nor reversion to type occurred during this procedure. It is probable that the biological differences between the "S antigen" of the Friedländer bacilli and the "S antigen" of Type II-S pneumococci are too great to induce the change of type.

RÉSUMÉ.

In this study efforts were made to determine the conditions under which the reversion of the avirulent R form to the virulent S form of Pneumococcus occurs. R strains have been encountered which reverted easily to their S prototype as well as R strains which appeared to be irreversible when treated by methods heretofore employed to induce the R→S change. By employing the method of Griffith many of the seemingly irreversible strains appeared to be converted into S forms.

During the course of the study the significance of secondary papillæ or daughter colonies which appeared within the R colonies was investigated. Daughter colonies often appeared during repeated transfers of a culture in specific immune serum broth, during repeated transfer on blood agar, and in an R strain residing in agar foci *in vivo*. In other words they appeared under conditions which are regarded as unfavorable to the bacteria. All strains of R pneumococci when treated under similar conditions did not reveal daughter colonies, and their

appearance even during the observations on a certain strain was inconstant. An exhaustive study of the conditions most favorable for the appearance of daughter colonies was not made.

During the course of the investigations the daughter colonies not only appeared within the R forms as shiny papillæ but appeared as individual separate large colonies which on account of their shiny surfaces were indistinguishable from the genuine S colonies. In some of the experiments there seemed to be a tendency of the daughter colonies to replace the R forms as if the daughter forms represented a more stable variety. The bacteria derived from the shiny daughter colonies were generally avirulent for mice, were serologically like the typical R strains, did not afford protection against infection with S forms, and had no demonstrable capsules. On one occasion the daughter forms reverted to the original S variety upon inoculation into animals. Although it is suggestive, the evidence is not sufficient to assume that the daughter form represents an intermediate stage during the R→S change. By applying the technic of Griffith it is apparently possible to convert certain R or daughter forms to S forms with equal facility.

Regarding the question of classification, a shiny daughter colony strain or a typical R strain if encountered separately would undoubtedly be assigned to Group IV pneumococci. However, the characteristics of R and daughter strains are typical and constant enough to justify a separate classification. Although the colony morphology of the 2 strains is different, the bacteria are serologically similar and are avirulent for mice. Certain strains have a tendency to revert spontaneously to their original prototypes, or by special treatment to revert or transform into other S types. Typical Group IV-S pneumococci may dissociate into the R form as well as Type I-, II-, or III-S strains.

SUMMARY.

A study was made of the behavior of R pneumococcus strains under various experimental conditions. It was found that daughter colonies frequently appeared among the R forms and in some instances tended to replace the typical R forms. The daughter strain produced colonies with shiny surfaces which were morphologically indistinguishable from

genuine S colonies although the characteristics of the bacteria comprising them conformed to the R variety. Both R and daughter forms which had resided for long periods of time *in vivo* have been converted into virulent forms. Strains of R pneumococci which seemed irreversible were apparently converted into S forms by applying the methods of Griffith.

A general consideration of the information derived during the recent experimental studies indicates that virulent S pneumococci may dissociate into the R form *in vivo*, that the R forms may be occasionally found in the sputum of pneumonia patients and also may reside or lie dormant *in vivo* for a considerable period of time. It has been shown that under favorable circumstances bacteria of the R form may spontaneously revert to the original S form or by special methods may even transform into S forms of another type.

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ANAPHYLAXIS WITH THE TYPE-SPECIFIC CARBOHYDRATES OF PNEUMOCOCCUS.

By OSWALD T. AVERY, M.D., and WILLIAM S. TILLETT, M.D.

(*From the Hospital of The Rockefeller Institute for Medical Research.*)

(Received for publication, October 9, 1928.)

Seligmann (1) (1911) was the first to test the capacity of the serum of pneumonia patients to sensitize guinea pigs to Pneumococcus. He inoculated the animals intravenously, with 5 to 6 cc. of serum, and 24 hours later infected them intravenously with suspensions of pneumococci. In no instance was anaphylactic sensitization induced by serum obtained before or after the crisis. Rosenow (2) (1911) found that guinea pigs treated with extracts of pneumococci became hypersensitive to a subsequent injection of these soluble products and that the sensitized animals exhibited a relatively slight yet definite immunity to infection. Clough (3) (1915) by the use of a protein extract of pneumococci produced anaphylactic sensitization in guinea pigs and found that the sensitized animals showed only slight evidence of any increased resistance to infection with virulent pneumococci. Weil and Torrey (4) (1916) injected guinea pigs with inactivated serum obtained from cases of pneumonia during the course of the disease and during convalescence. 4 to 6 days later the animals were tested by the Dale method to determine whether they had acquired an increased hypersensitiveness to autolytic extracts of pneumococci. A positive reaction occurred with the serum of all but two of twenty patients. The sensitizing action when present was demonstrable in serum obtained during the febrile period of the disease and in only two instances did it persist in serum obtained during convalescence. The serum of normal individuals and of patients suffering from diseases other than pneumonia failed to sensitize control animals to the same bacterial extracts. In guinea pigs actively sensitized by repeated intraperitoneal injections of large amounts of heat-killed pneumococci, Zinsser and Mallory (5) (1924) succeeded in obtaining, with alkaline extracts of pneumococci, uterine reactions which they considered comparable in every way to those obtained in anaphylactic sensitization with non-bacterial proteins. They also demonstrated that by passive sensitization, uterine reactions, analogous to those elicited in true protein anaphylaxis, can be obtained in guinea pigs with soluble products of Pneumococcus. However, they found that active sensitization proved difficult, and passive sensitization was never very satisfactory and often impossible.

In guinea pigs previously immunized by killed and living virulent pneumococci, Mackenzie (6) (1924) produced fatal anaphylactic shock by the intravenous in-

jections of amounts of culture filtrate quite innocuous for control animals. While definite anaphylactic response to extracts of the bacterial protein was never elicited in animals similarly immunized, positive results, on the other hand, were obtained by the Dale method when the sensitized uterine muscle was exposed to pneumococcus protein. In this latter type of experiment, however, culture filtrates were found unsuitable because of the narrow margin between the amount necessary to elicit the anaphylactic response, and the quantity primarily toxic for the unsensitized control. In studying the significance of anaphylaxis in pneumococcus immunity Mackenzie observed that although during the course of immunization, the animals developed a high degree of active immunity and that their serum conferred on other guinea pigs passive protection against infection, at no time did the serum show the presence of demonstrable agglutinins for Pneumococcus or precipitins for its soluble derivatives. On the basis of the experimental results, he concludes that there is no significant relationship between anaphylaxis to pneumococcus protein and resistance to infection, since the latter could be demonstrated both in the presence and in the absence of the former.

Previous studies on anaphylaxis with Pneumococcus have dealt with the action of cell solutions and autolytic extracts containing admixtures of bacterial protein and other soluble products, which are often primarily toxic. As a result of the more recent chemo-immunological studies carried out in this laboratory, preparations of the specific carbohydrates of Pneumococcus were available in purified form. These soluble specific substances are free from protein and are devoid of primary toxic properties. In view of their increasing significance in the processes of immunity, it seemed important to determine whether the protein-free polysaccharides isolated from the specific types of Pneumococcus actively participate in the mechanism of bacterial anaphylaxis.

The present paper records the results of attempts by the use of these bacterial carbohydrates, (1) to induce active sensitization in guinea pigs, (2) to produce fatal anaphylactic shock in animals passively sensitized with antibacterial serum of the homologous type, (3) to define the reactions in terms of the chemical and biological specificity of the reagents employed.

EXPERIMENTAL.

Chemical Properties of Pneumococcus Polysaccharides.—The chemical nature of the soluble specific substances derived from each of the three types of Pneumococcus has been described in preceding papers

(7). The essential properties of the particular preparations used in the present experiments are briefly summarized in Table I.

As previously pointed out, all these substances possess the chemical properties of complex sugars; they contain no phosphorus, nor sulfur and they give none of the usual protein color tests. The Type II and Type III substances are nitrogen-free. The Type I substance differs from the other two in containing nitrogen as an apparently essential component; one-half of this nitrogen is present in the amino form. Despite the presence of nitrogen, the substance fails to give

TABLE I.

Soluble Specific Substances of Pneumococcus, Types I, II, III. Carbohydrate-Haptens.

Type	Preparation No.	$[\alpha]_D$	Acid equivalent	N	Reducing sugar on hydrolysis*	Highest dilution giving precipitate with anti-pneumococcus horse serum
				per cent	*per cent*	
I	59	+304	—	5.0†	32.0	1:6,000,000
II	26-A	+72.8	1252	0.0‡	68.2	1:5,000,000
III	53-A	−34	338	0.0‡	70.0	1:5,000,000

* Calculated as glucose.

† Amino N, 2.5 per cent.

‡ Analyzed by the Pregl micro Kjeldahl method, using 50 mg. of substance.

any of the protein color reactions. Quantitative solutions of the substances were made in salt solution and sterilized by heat.

Active Sensitization.—In the present investigation no attempt has been made to induce active anaphylaxis in animals by sensitization with the whole bacterial bodies. The work is confined solely to a study of the anaphylactic response of animals to the type-specific carbohydrate of Pneumococcus.

Guinea pigs of an average weight of 350 gm. were used. They were divided into three groups of six animals each. Of six guinea pigs in Group 1, two received a single initial dose of 50 mg., two 10 mg. and two 5 mg. of the specific carbohydrate derived from Type I pneumococci. The same number of animals in Groups 2 and 3 received similar amounts of the corresponding carbohydrates of organisms of Type II and Type III. 21 days after this initial treatment all the

TABLE II.

Active Anaphylaxis with the Specific Carbohydrates of Pneumococcus.

Type I—Carbohydrate (1).

Guinea pig No.	Sensitizing dose i.p.	Interval	Shocking dose i.v.	Result
	mg.	*days*	*mg.*	
1	5	21	10	No reaction
2	5	21	1	" "
3	10	21	5	" "
4	10	21	1	" "
5	50	21	10	" "
6	50	21	1	" "

Type II—Carbohydrate (2).

Guinea pig No.	Sensitizing dose i.p.	Interval	Shocking dose i.v.	Result
	mg.	*days*	*mg.*	
7	5	21	10	No reaction
8	5	21	1	" "
9	10	21	10	" "
10	10	21	1	" "
11	50	21	10	" "
12	50	21	1	" "

Type III—Carbohydrate (3).

Guinea pig No.	Sensitizing dose i.p.	Interval	Shocking dose i.v.	Result
	mg.	*days*	*mg.*	
13	5	21	10	No reaction
14	5	21	1	" "
15	10	21	10	" "
16	10	21	1	" "
17	50	21	10	" "
18	50	21	1	" "

1 = Preparation 59 (see Table I).
2 = Preparation 26-A (see Table I).
3 = Preparation 53-A (see Table I).
i.p. = intraperitoneal.
i.v. = intravenous.

animals of each group were injected intravenously with the second or shocking dose of from 1 to 10 mg. of the carbohydrate of the homologous type. The results are presented in Table II.

Attempts to induce active anaphylactic sensitization with pneumococcus polysaccharides were uniformly negative, as evidenced by the failure of the animals to react to a second injection of the homologous substance (Table II). The complete absence of the signs and symptoms of anaphylaxis in the guinea pigs, treated in the manner described, affords convincing evidence that the type-specific carbohydrate of Pneumococcus, in the dissociated and purified state, is by itself devoid of the property of inducing active sensitization. The criticism may be made, that either the sensitizing or the intoxicating dose or both may not have been adequate to elicit the response; that the interval between the two injections may not have been optimal for the development of sensitization. Unfortunately, the answer to these questions would require more elaborate experiments than the available amounts of the chemically purified substances permit. However, the maximal sensitizing dose of 50 mg. of Type I polysaccharide represents the chemical yield from approximately 5 liters of broth culture, and the amount recoverable from about 500 cc. of culture in the case of the Type III substance. In terms of the effective dosage in protein anaphylaxis, the amounts of bacterial carbohydrate employed both for sensitization and intoxication cover a sufficiently wide range to yield at least some evidence of activity were these substances possessed of sensitizing properties. Moreover, as described later in this paper, the amounts given at the time of the second injection were from 40 to 400 times greater than the minimum dose required to produce fatal anaphylactic shock in animals passively sensitized with antibacterial serum of the homologous type. It also seems unlikely that the failure to elicit the anaphylactic response was due to the lack of an appropriate interval between the two injections since the time chosen, namely 21 days, is usually optimal for the development of maximal sensitivity in the case of true antigens. Therefore, under the experimental conditions, the conclusion seems justified that, in the protein-free form, the immunologically type-specific polysaccharides of Pneumococcus are devoid of the property of inducing anaphylactic sensitization in the animal body.

Passive Sensitization.—The lack of sensitizing action, as tested in the foregoing experiments, adds further evidence in support of the view that the specific carbohydrates of Pneumococcus, when chemically purified, are devoid of antigenic properties. The fact, however, that in the protein-free form they retain unimpaired the function of combining specifically with antibacterial antibodies of the homologous type, led to attempts to ascertain whether these bacterial sugars are capable of provoking anaphylactic shock in animals passively sensitized with specific precipitating serum. Guinea pigs, previously injected with antipneumococcus serum containing a high titer of type-specific precipitins, were later tested to determine whether they had become hypersensitive to the specifically precipitable polysaccharide.

Antibacterial sera of Types I, II and III were produced by the intravenous injection of rabbits with heat-killed suspensions of pneumococci according to the method of Cole and Moore (8). It has long been recognized that it is possible to elicit specific antibodies to Type III in only an occasional rabbit. Tillett (9) has recently shown that this peculiarity in the immune response to Type III is due, in part, to variation in the resistance of individual rabbits and is also dependent upon essential differences in the antigenic complex of organisms of this type. However, by special procedures to be recorded later, the antibacterial rabbit sera employed in these experiments contained a relatively high titer of precipitins for the type-specific polysaccharides. Antipneumococcus horse serum, prepared by the method of Wadsworth, was also used.[1] The immune horse serum of the three specific types contained a higher concentration of carbohydrate-precipitating antibodies than did the corresponding immune rabbit serum. This fact is important in view of the differences in the results obtained with sera of different animal origin.

1. Immune Rabbit Serum.—Guinea pigs weighing 350 to 400 gm. were passively sensitized by a single intraperitoneal injection of antipneumococcus serum. At intervals varying from 1 to 7 days after the administration of the serum, a known amount of a quantitative solu-

[1] The antipneumococcus horse serum (Types I, II and III) employed in this study was furnished through the courtesy of Dr. Augustus B. Wadsworth, Director of Laboratories, New York State Department of Health.

tion of the polysaccharide of the corresponding type was injected into one of the superficial veins of the leg.

TABLE III.

Passive Anaphylaxis to the Specific Carbohydrate of Pneumococcus Type I in Guinea Pigs Sensitized with Type I Antipneumococcus Rabbit Serum.*

Guinea pig No.	Type I antipneumococcus serum (rabbit) i.p.	Time interval	Type I carbohydrate i.v.	Symptoms	Result	Autopsy
	cc.	*hrs.*	*mg.*			
1	1	24	1.0	Typical	†3 min.	Typical
2	1	24	0.05	"	†4 "	"
3	1	24	0.05	"	†5 "	"
4	1	24	0.025	"	†4 "	"
5	1	24	0.005	"	†4 "	"
6	1	24	0.005	"	†3 "	"
7	1	24	0.002	Slight respiratory embarrassment	Recovery	—
8	1	48	0.025	Typical	†5½ min.	Typical
9	1	48	0.025	"	†3½ "	"
10	1	48	0.025	"	†5 "	"
11	1	48	0.010	Scratches, coughs, bucks, dyspnea	Recovery 5 min.	—
12	1	72 *days*	0.025	Typical	†3½ min.	Typical
13	1	7	3.0	"	†3½ "	"
14	1	7	3.0	Scratches, coughs, falls on side	Recovery 6 min.	—
15	1	7	2.0	Slight symptoms	"	—
16	1	7	1.0	No symptoms	No reaction	—

* Preparation 59 (see Table I).
† Death of animal.
i.p. = intraperitoneal.
i.v. = intravenous.

The results of the experiments on passive anaphylaxis are given in Tables III, IV and V. The data presented in Table III show that the protein-free carbohydrate, derived from Pneumococcus Type I,

TABLE IV.

Passive Anaphylaxis to the Specific Carbohydrate of Pneumococcus Type II in Guinea Pigs Sensitized with Type II Antipneumococcus Rabbit Serum.*

Guinea pig No.	Type II antipneumococcus serum (rabbit) i.p.	Time interval	Type II carbohydrate i.v.	Symptoms	Result	Autopsy
	cc.	*hrs.*	*mg.*			
1	2	24	1.0	Typical	†2½ min.	Typical
2	2	24	0.5	"	†3 "	"
3	2	24	0.1	"	†4 "	"
4	2	24	0.03	"	†5 "	"
5	2	24	0.025	"	†4½ "	"
6	2	24	0.01	Slight dyspnea	Recovery	—

* Preparation 26-A (see Table I).
† Death of animal.
i.p. = intraperitoneal injection.
i.v. = intravenous injection.

TABLE V.

Passive Anaphylaxis to the Specific Carbohydrate of Pneumococcus Type III in Guinea Pigs Sensitized with Type III Antipneumococcus Rabbit Serum.*

Guinea pig No.	Type III antipneumococcus serum (rabbit) i.p.	Time interval	Type III carbohydrate i.v.	Symptoms	Result	Autopsy findings
	cc.	*hrs.*	*mg.*			
1	3	24	1.0	Cough, bucking, dyspnea severe	Severe reaction, recovery	—
2	3	24	1.0	Typical	†3½ min.	Typical
3	3	48	1.0	"	†4 "	"
4	3	48	0.5	"	†2½ "	"
5	3	72	2.0	"	†3 "	"
6	3	72	1.0	"	†3½ "	"

* Preparation 53-A (see Table I).
† Death of animal.
i.v. = intravenous injection.
i.p. = intraperitoneal injection.

in amounts as small as 0.005 mg., produced rapid and fatal anaphylactic shock in guinea pigs passively sensitized with 1 cc. of immune rabbit serum Type I. The symptoms and autopsy findings were in every respect identical with those characteristic of true protein anaphylaxis. The reaction was readily elicited 24 hours after administration of the sensitizing serum and could still be provoked 3 to 7 days later; as the time interval increased, however, larger amounts of the toxigenic substance were required and the response became progressively less. In the case of the nitrogen-free polysaccharides of Pneumococcus Type II and Type III, typical and fatal anaphylactic

TABLE VI.

Type Specificity of the Anaphylactic Response to Pneumococcus Carbohydrate in Guinea Pigs Sensitized with Antipneumococcus Rabbit Serum.

Guinea pig No.	Antipneumococcus serum (rabbit)		Time interval	Heterologous carbohydrate		Result	20 min. later same animals injected with homologous carbohydrate		Result	Autopsy findings
	Type	*cc.*	*hrs.*	*Type*	*mg.*		*Type*	*mg.*		
1	I	1.0	48	II	0.05	No reaction	I	0.025	†5 min.	Typical
2	II	1.0	24	I	1.0	" "	II	0.03	†3½ "	"
3	II	2.0	72	I	1.0	" "	II	0.025	†5 "	"
4	II	2.0	72	III	1.0	" "	II	0.5	†4 "	"
5	II	1.0	74	I	0.25	" "	II	0.25	†3½ "	"

The sensitizing serum was given intraperitoneally; the carbohydrates were injected intravenously.

† Death of animal.

shock was produced in passively sensitized animals by the injection of 0.025 mg. and 0.5 mg. of the substances respectively.

The bacterial sugars themselves are not primarily toxic. Normal control guinea pigs injected with large amounts have in no instance exhibited any toxic symptoms.

The specificity of the reaction is shown in Table VI. Guinea pigs sensitized with the immune serum of one type are wholly unaffected by the intravenous injection of the carbohydrate of a heterologous type. These same animals, however, succumb promptly with all the characteristic symptoms of anaphylaxis, when subsequently injected

with a fraction of a milligram of the polysaccharide corresponding in type to that of the immune serum with which they were originally sensitized.

The results of the intravenous injection of minute quantities of these specific polysaccharides into serum-sensitized guinea pigs, establish the fact that passive anaphylaxis can be produced by the

TABLE VII.

Absence of Anaphylactic Response to the Specific Carbohydrate of Pneumococcus Type I in Guinea Pigs Sensitized with Type I Antipneumococcus Horse Serum.

Guinea pig No.	Type I antipneumococcus serum (horse)	Time interval	Type I carbohydrate	Symptoms	Result
	cc.	*hrs.*	*mg.*		
1	4	24	2.5	Slight coughing, no other symptoms	Survival
2	1	24	2.5	None	"
3	4	24	1.0	Occasional cough, no other symptoms	"
4	1	24	1.0	None	"
5	4	24	0.5	Slight abdominal breathing	"
6	1	24	0.05	None	"
7	1	24	0.025	"	"
8	1	24	0.025	"	"
9	4	48	1.0	"	"
10	4	48	0.01	"	"
11	4	72	2.0	"	"
12	4	72	1.0	"	"
13	4	72	0.01	"	"

Guinea Pigs 1 to 6 received immune horse serum Lot 345 (Albany).
Guinea Pigs 7 to 12 received immune horse serum Lot 7 P_8 (New York).
Sensitizing serum was injected intraperitoneally.
Carbohydrate was injected intravenously.

protein-free carbohydrates of Pneumococcus, and that the reactions are type-specific. Moreover, the carbohydrates of Types II and III have been chemically purified to the point where they no longer contain nitrogen. The Type I substance, even when subjected to equally vigorous methods of purification, always retains a residue of nitrogen which appears to be an essential component of the carbo-

hydrate molecule, since it cannot be split off without loss of immunological specificity. Despite the presence of nitrogen this substance gives none of the protein color tests. The experimental evidence, therefore, is that the protein-free carbohydrates—the type-specific haptens—of Pneumococcus produce anaphylactic shock in animals passively sensitized with the precipitating serum of rabbits immunized with the bacterial cells from which these substances are derived.

TABLE VIII.

Absence of Anaphylactic Response to the Specific Carbohydrate of Pneumococcus Type II in Guinea Pigs Sensitized with Type II Antipneumococcus Horse Serum.

Guinea pig No.	Type II anti-pneumococcus serum (horse)	Time interval	Type II carbohydrate	Symptoms	Result
	cc.	*hrs.*	*mg.*		
1	1	24	1.0	None	Survival
2	1	24	0.25	"	"
3	5	48	2.0	"	"
4	5	48	1.0	"	"
5	5	72	2.0	"	"
6	5	72	1.0	"	"

Guinea Pigs 1 to 4 received intraperitoneally immune serum of Horse 92.
Guinea Pigs 5 and 6 received intraperitoneally immune serum of Horse 107.
Carbohydrate injected intravenously.

2. Immune Horse Serum.—The successful experiments on passive anaphylaxis, recorded above, were all carried out on guinea pigs sensitized with immune rabbit serum. On the other hand, attempts to render guinea pigs passively anaphylactic by the use of antipneumococcus horse serum were uniformly unsuccessful (Tables VII to IX).

The absence of the anaphylactic response to pneumococcus carbohydrate in guinea pigs passively sensitized with immune horse serum, when contrasted with the invariably fatal reaction induced in animals sensitized with immune rabbit serum, seemed at first inexplicable; it was all the more surprising in view of the fact that the horse serum

in vitro precipitated the type-specific carbohydrate in higher dilution than did the corresponding serum of the immune rabbits.

This difference in the behavior of two sera, each of different animal origin but both containing precipitating antibodies of the same type, may possibly be explained, in part at least, by the fact, first noted by Zinsser and Parker (10), that antipneumococcus horse serum contains a substance which interferes with the fixation of complement. The same phenomenon has been observed in this laboratory by Lancefield (11) and by Vollmond (12), who found that in a system containing the purified, type-specific carbohydrate (hapten) of Pneumococcus

TABLE IX.

Absence of Anaphylactic Response to the Specific Carbohydrate of Pneumococcus Type III in Guinea Pigs Sensitized with Type III Antipneumococcus Horse Serum.

Guinea pig No.	Type III anti-pneumococcus serum (horse)*	Time interval	Type III carbohydrate	Symptoms	Result
	cc.	*hrs.*	*mg.*		
1	5	24	2.0	None	Survival
2	5	24	1.0	"	"
3	5	48	2.0	"	"
4	5	48	1.0	"	"
5	5	72	2.0	"	"
6	5	72	1.0	"	"

* Immune horse serum (Albany, Lot 68).

complement fixation takes place in the presence of homologous immune rabbit serum but does not occur when immune horse serum is used.

When the nucleoprotein of the cell, instead of the carbohydrate hapten, was used in the test, both Lancefield and Vollmond found that complement fixation occurred with the immune sera of both species.

The fact that the anaphylactic response to the specific hapten does not occur in guinea pigs passively sensitized with antipneumococcus horse serum may be referable to the presence in the serum of the same substance which inhibits the fixation of complement with

pneumococcus polysaccharide. There is a certain amount of evidence in favor of the view that complement actively participates in the mechanism of anaphylaxis. The chief facts supporting this view may be briefly summarized as follows: (1) the diminution of complement during anaphylactic shock (13); (2) the prevention of anaphylaxis by hypertonic salt solution which is also known to inhibit the fixation of complement *in vitro* (14); (3) the absence of anaphylactic response to protein in guinea pigs after depletion of their complement by the injection of sensitized sheep cells (15); (4) the action of complement in the formation of anaphylotoxins (16, 17). It may be possible, therefore, that the substance in antipneumococcus horse serum which is known to interfere with the fixation of complement in the test-tube, may also inhibit the action of complement in the animal body and thus prevent the anaphylactic reaction when the type-specific hapten (carbohydrate) of Pneumococcus is injected into guinea pigs sensitized with immune horse serum.

DISCUSSION.

Tomcsik (18) isolated from *B. lactis aerogenes* a soluble, specific substance consisting largely of carbohydrate which failed to give the protein reactions and which, despite repeated attempts at further purification, contained 0.9 per cent of nitrogen. In passively sensitized guinea pigs, the injection of 0.02 mg. of this substance induced fatal anaphylactic shock, while doses of 1 mg. failed to cause any reaction in unsensitized control animals. By the Schultz-Dale method, positive uterine reactions were obtained when minute amounts of the substance were added to the reaction bath, and distinct contractions were observed in dilutions as high as 1:20,000,000. Tomcsik and Kurotchkin (19) in a subsequent paper have reported similar results with the specific carbohydrate material isolated from *B. lactis aerogenes*, the pneumobacillus and a yeast. Lancefield (20) has recently isolated from streptococci carbohydrate material which produces anaphylactic shock in guinea pigs passively sensitized with antistreptococcus serum. Because of the presence of small amounts of nitrogen in the products they used, none of these authors felt justified in concluding that the bacterial carbohydrate alone produced the anaphylactic shock.

The bacterial carbohydrates used in the present study represent the most highly purified preparations isolated in this laboratory by Heidelberger and Goebel from type-specific strains of Pneumococcus. The Type II and Type III substances are nitrogen-free, and the Type I substance, despite the presence of apparently essential nitrogen (5 per cent), is protein-free, as far as can be determined by chemical analysis. These bacterial derivatives belong to the unique group of immunologically specific substances which Landsteiner (21) called haptens. When split off from the complex antigens of which they form a part, these substances are no longer antigenic but still retain the property of binding the type-specific antibodies produced by the whole antigen. As previously pointed out, the carbohydrate haptens of Pneumococcus are immunologically identical with the bacterial products originally referred to as the soluble specific substance.

Considering the carbohydrate nature and the non-antigenic properties of pneumococcus haptens it is not surprising that they fail to induce active anaphylactic sensitization. In passive anaphylaxis, the results obtained with the carbohydrates of Pneumococcus demonstrate that protein-free and even non-nitrogenous substances of the nature of bacterial polysaccharides are capable of inducing anaphylactic shock in guinea pigs sensitized with antibacterial serum of the homologous type.

Previous studies have shown that the reactions of precipitation and complement fixation with these substances occur in immune rabbit serum and are type-specific. The present work demonstrates that the anaphylactic reaction also occurs when minute amounts of the same carbohydrate haptens are injected into animals passively sensitized with immune rabbit serum. However, an interesting difference in the occurrence of these three immune reactions has been found to exist when antipneumococcus *horse* serum is used instead of *rabbit* serum. Although these substances are equally precipitable by the immune sera of both species, they combine in the reaction of complement fixation in the test-tube and induce passive anaphylaxis in the animal body when immune rabbit serum is used, and they fail to react when immune horse serum is employed. It is not possible at present to state whether the same inhibiting substance in antipneumococcus horse serum which interferes with the fixation of complement in a

system containing the specific carbohydrate also prevents the occurrence of anaphylactic shock by interfering with the action of complement in the animal body. The phenomenon seems peculiar to the carbohydrate haptens since it does not occur when the protein fraction of the cell is used with the same immune horse serum. The elucidation of this phenomenon requires further study. The present paper deals only with the facts as they relate to the reactions induced by the non-protein carbohydrates. The facts appear sufficiently established to justify the conclusion that anaphylactic shock can be induced by the injection of minute amounts of the type-specific carbohydrates of Pneumococcus in guinea pigs passively sensitized with homologous antipneumococcus rabbit serum.

CONCLUSIONS.

1. The type-specific carbohydrates (haptens) of Pneumococcus Types I, II and III, when isolated in protein-free form, are devoid of the property of inducing active anaphylactic sensitization in guinea pigs.

2. The bacterial carbohydrates of Pneumococcus, of which the Type II and Type III substances are nitrogen-free, produce rapid and fatal anaphylactic shock in guinea pigs passively sensitized with the precipitating serum of rabbits immunized with pneumococci of the homologous type; the reactions induced are type-specific.

3. In contrast to the positive results with *immune rabbit serum*, there is a complete absence of anaphylactic response to pneumococcus carbohydrate in guinea pigs passively sensitized with *antipneumococcus horse serum.*

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A STUDY OF PNEUMOCOCCUS AUTOLYSIS.

By WALTHER F. GOEBEL, Ph.D., and OSWALD T. AVERY, M.D.

(*From the Hospital of The Rockefeller Institute for Medical Research.*)

(Received for publication, October 15, 1928.)

Although it has long been recognized that one of the distinguishing characters of Pneumococcus is the ease with which the cell undergoes autolysis, relatively little is known of the chemical changes accompanying this process.

Rosenow (1) measured small increases in amino nitrogen in pneumococcus autolysates and found that the appearance and subsequent disappearance of toxic substances in saline suspensions of pneumococci and other bacteria, when kept at 37°C., are associated with proteolysis. Jobling (2) and his collaborators, in their studies on ferment action, concluded from the results of determinations of the amino nitrogen content of pneumococcus autolysates, that the solution of bacteria is not to be confused with proteolysis. They observed also that leucoprotease acting on pneumococci results in lower cleavage products than those obtained with the autolytic enzymes of Pneumococcus itself; and that the products of protease digestion are less toxic than the autolytic products, presumably because, in the former instance, proteolysis is carried beyond the toxic stage.

Neufeld (3) was the first to show that Pneumococcus is soluble in bile and that the salts of cholic acid have the same action as bile itself. The solvent action of bile Neufeld attributes to the marked swelling and precipitation of the cell membrane whereby it no longer affords the bacterial body the same protection as does the normal membrane.

Lord and Nye (4) concluded that solution of Pneumococcus results from the action of the intracellular enzymes, and that this action is facilitated by the addition of bile. More recently Atkin (5), investigating the relationship of autolysis to bile solubility, states that "the solution of the pneumococcus in bile consists in an acceleration by this substance of the normal autolytic process."

Avery and Cullen (6), studying the action of the intracellular enzymes of Pneumococcus on foreign substrates, found that bacterial extracts containing these active agents brought about cleavage of various carbohydrates, esters, and protein substances.

The present investigation includes the study of the enzymes of Pneumococcus not on foreign substrates, but on the native substances

of the cell itself. The experiments fall into two groups: (1) those which have to do with the chemical changes accompanying autolysis as it occurs spontaneously in suspensions and bile solutions of living cells; (2) those which deal with the action of these same enzymes when, freed from the cell, they are allowed to act upon suspensions of heat-killed pneumococci, or upon emulsions of the alcohol-soluble lipoids extracted from them. These experiments were undertaken not only to acquire additional information concerning the nature of the autolytic process, but also with hope that an analysis of the chemical changes taking place during autolysis might furnish some clue to the nature of change, which, following cell lysis, results in the diminution or loss of the type-specific antigenicity of the dissolved material. For, as has been pointed out in preceding papers, cell disintegration, whether brought about by physical means (freezing and thawing), by chemical agents (bile or alkali), or by enzyme action (autolysis), is generally accompanied by partial or complete loss of the capacity on the part of the dissolved material to stimulate in the animal body the type-specific antibodies which characterize the immune response to the intact cell.

Previous studies have dealt in detail with the chemical nature and immunological significance of the so called soluble specific substance of Pneumococcus upon which the type specificity of the cell has been found to depend (7). It need only be pointed out here, that chemically these type-specific substances fall into the class of carbohydrates and that immunologically they belong to that important group of specifically reactive but non-antigenic substances which Landsteiner has named "haptens." In the protein-free form, they all possess in common the properties of polysaccharides, but individually they exhibit differences in chemical and immunological reactions which are specific and constant for each of the three types of Pneumococcus. When isolated from the cell in purified form, these complex sugars (haptens) retain the property of specific precipitation in antibacterial sera of the homologous type, but lose completely the antigenic function of inciting antibody formation. The fact that, in the natural state in which they occur in the bacterial cell, these carbohydrate substances are not only highly type-specific but extraordinarily effective as antigens, and the additional fact that they lose their antigenic properties

to a greater or less extent whenever cell dissolution occurs, afford strong evidence that the encapsulated cell contains a complex antigen in which the type-specific carbohydrate (hapten) is combined with some other constituent, possibly protein, to form the intact antigen. The nature of the substance which confers antigenicity upon the hapten, the character of the antigenic union, and the conditions determining antigenic dissociation *in vitro* and *in vivo* are questions as yet unsolved. A direct study of the chemistry of this complex antigen, and the solution of the problems relating to it involve technical difficulties which are not easily overcome. However, it seemed possible that a detailed investigation of the chemical changes accompanying autolysis might indirectly at least provide a lead, since the processes of cell autolysis and antigenic dissociation are intimately associated if not causally related.

EXPERIMENTAL.

1. Proteolysis as Measured by the Increase of Amino and Non-Coagulable Nitrogen in Pneumococcus Autolysates.

The growth from 1.5 liters of broth cultures of pneumococci was collected by centrifugation and washed once with M/150 phosphate buffer of the desired hydrogen ion concentration. After recentrifugation the bacteria were suspended in 30 cc. of the same buffer. The suspension was divided into two equal portions. One part, which served as the control, was immediately inactivated by heating at 100°C. for 10 minutes. The other part containing the active cells was placed at 37°C. to autolyze. After 48 hours incubation, complete disintegration of the bacteria occurred as shown by the fact that in stained preparations only Gram-negative detritis remained. In these experiments, aseptic technique was employed throughout, and no preservatives were added to the bacterial deposits.

The heated control material (Tube C) and the active autolysate (Tube A) were each analyzed for amino and total nitrogen. Duplicate determinations were made in each instance. The method of Van Slyke (8) was employed for determination of amino nitrogen, 5 cc. of bacterial suspension or autolysate being used for each analysis. Total nitrogen was estimated by the Pregl (9) micro Kjeldahl method, 2 cc. of material being used for analysis. Non-coagulable nitrogen was estimated by the method described below. A summary of these experiments is given in Tables I and II. The experiments summarized in Table I were made on pneumococci of different types with buffer solutions of different hydrogen ion concentrations. In Experiments 4, 9, and 11, Type I pneumococcus was employed. The bacteria were washed once in M/150 buffer before resuspending them in the same buffer for autolysis. Tube C, the control tube, was heated 10 minutes

TABLE I.

Proteolysis Accompanying Autolysis of Pneumococcus as Measured by the Increase in Amino Nitrogen.

Exp. No.	Pneumococcus type	Tube	pH	Total nitrogen	Amino nitrogen	N in amino form	Increase in amino nitrogen
				mg. per cc.	*mg. per cc.*	*per cent*	*per cent*
4	I	C*	7.5	0.42	0.042	10.0	—
		A		0.43	0.060	13.9	39.0
9	I	C	7.5	0.47	0.050	10.6	—
		A		0.44	0.065	14.7	38.7
11	I	C	5.5†	0.29	0.030	10.4	—
		A		0.34	0.044	12.9	24.1
22	III	C	7.5	0.31	0.027	8.7	—
		A		0.30	0.035	11.6	33.3
6	III	C	6.5	0.58	0.064	11.0	—
		A		0.56	0.080	14.3	30.0
7	III	C	5.5	0.43	0.049	11.4	—
		A		0.41	0.059	14.4	26.2
8	III	C	NaCl‡	0.47	0.053	11.3	—
		A		0.56	0.067	11.9	5.3
15	II	C	7.5	0.49	0.029	5.9	—
		A		0.49	0.037	7.5	27.1
20	II	C	7.5	0.52	0.059	11.3	—
		A		0.50	0.068	13.6	20.3
16	II	C	6.5	0.51	0.053	10.4	—
		A		0.51	0.061	12.0	15.4
17	II	C	6.5	0.53	0.052	9.8	—
		A		0.51	0.060	11.8	20.4
19	II	C	NaCl	0.51	0.059	11.5	—
		A		0.51	0.073	14.3	24.4
18	II	C	NaCl	0.45	0.047	10.4	—
		A		0.45	0.064	14.2	36.5

* C = control, heat-inactivated. A = active, autolysate.

† Acetic acid-sodium acetate buffer.

‡ Isotonic salt solution.

TABLE I—*Concluded.*

Exp. No.	Pneumococcus type	Tube	pH	Total nitrogen	Amino nitrogen	N in amino form	Increase in amino nitrogen
				mg. per cc.	*mg. per cc.*	*per cent*	*per cent*
28	II	C	7.5	0.25	0.019	7.6	—
		A		0.28	0.026	9.3	22.4
31	II	C	7.5	0.48	0.045	9.4	—
		A		0.48	0.054	11.3	20.2
29	II	C	6.5	0.38	0.030	7.9	—
		A		0.38	0.039	10.3	30.4
30	II	C	6.5	0.31	0.028	9.0	—
		A		0.31	0.035	11.3	25.5
32	II	C	5.5	0.36	0.026	7.2	—
		A		0.36	0.028	7.8	8.3
33	II	C	5.5	0.31	0.020	6.5	—
		A		0.31	0.022	7.1	9.2
27	II	C	NaCl	0.20	0.014	7.0	—
		A		0.21	0.018	8.6	22.9

at 100°C., and was placed on ice. Tube A, the autolysate, was incubated for 48 hours at 37°C. In Experiments 22, 6, 7, and 8 the Type III organism was used in buffer solutions of the same molar concentration. In Experiments 15 to 18, however, the Type II organism and M/15 buffer were used. In Experiments 28 to 27 the bacteria were washed three times with M/15 buffer before incubating at 37°C.

A careful consideration of the data in Tables I and II reveals that the autolysis of pneumococci is accompanied by an increase in the amino and non-coagulable nitrogen of some substance in the bacterial suspension. Although various experiments were carried out with pneumococci of different types, and buffer solutions of different molar and hydrogen ion concentrations, in each experiment, regardless of these variations, a definite increase was always observed. At first it was thought that this increase in amino and non-coagulable nitrogen might be due to the action of the intracellular proteolytic enzymes on peptone adsorbed by the organism from the meat infusion broth, but experiments on pneumococci washed as often as three times before

autolysis showed an increase of the same order of magnitude as that observed when the cells were washed only once. Furthermore, when a substrate of heat-killed pneumococci is treated with a potent pneumococcus extract containing the active intracellular enzymes, the cells undergo lysis (10), and there occurs an increase in amino and non-coagulable nitrogen of the same order of magnitude as that observed in true autolysis (see Table II, Experiment K).

TABLE II.

Proteolysis Accompanying Autolysis of Pneumococcus as Measured by the Increase in Amino and Non-Coagulable Nitrogen.

Exp. No.	Pneumococcus type	Tube	pH	Total nitrogen	Non-coagulable N	Amino nitrogen	N in amino form	N in non-coagulable form	Increase in amino N	Increase in non-coagulable nitrogen
				mg. per cc.	*mg. per cc.*	*mg. per cc.*	*per cent*	*per cent*	*per cent*	*per cent*
40	II	C	6.5	0.44	0.17	0.042	9.5	38.6	—	—
		A	6.5	0.39	0.23	0.045	11.5	59.0	21.1	52.9
41	II	C	6.5	0.40	0.15	0.042	10.5	37.5	—	—
		A	6.5	0.36	0.20	0.045	12.5	55.4	19.1	47.8
42	II	C	NaCl	0.20	0.05	0.024	12.0	25.0	—	—
		A	NaCl	0.16	0.07	0.023	14.4	43.7	20.0	75.0
K*	III	C	7.5	0.74	0.21	0.098	13.2	28.4	—	—
		A	7.5	0.74	0.36	0.122	16.5	48.7	25.0	71.5

* Heat-killed pneumococci to which inactive (Tube C) and active (Tube A) bacteriolytic enzymes were added and both tubes incubated 48 hours at 37°C.

During autolysis of Pneumococcus, about 20 per cent of the total nitrogen is changed from the coagulable to the non-coagulable form, and about 2 per cent of total nitrogen from the non-amino to the amino form. It seems, therefore, that this proteolysis is of the nature of a peptic digestion in which the protein is broken down to the albumose stage, rather than of a tryptic digestion in which the ratio of amino to total nitrogen is invariably greater than that found in pneumococcus autolysates.

Proteolysis is apparently due not to the action of the bacterial

enzymes on a foreign substrate, but to their action on the parent substances of the cell itself. It seems difficult to explain the great fluctuations in the increase of amino and non-coagulable nitrogen, when conditions of experimentation were apparently kept constant. It must be remembered, however, that bacterial suspensions behave not as solutions of inert chemical substances, but as aggregates of living cells where variations of every sort may be encountered. Even a single disturbance, such as a slight change in the proportion of substrate to the content of cellular enzyme could account for these fluctuations.

2. Proteolysis in Pneumococcus Solutions Containing Sodium Desoxycholate.

The growth from 3 liters of broth culture of Pneumococcus was collected as described above. The washed bacteria were suspended in 100 cc. of M/15 phosphate buffer solution and the suspension was divided into four equal parts. The first part, which served as a control, was immediately heated for 10 minutes at 100°C. The second part was allowed to autolyze at 37°C. for 48 hours, during which time complete disintegration of the bacterial cells occurred. The third and fourth parts were cooled to 0°C., and to each were added 1.5 cc of 10 per cent sodium desoxycholate. After complete solution of the bacteria in the cold, one portion was immediately heated for 10 minutes at 100°C. to inactivate the autolytic ferments; the other portion, unheated, was placed in the incubator at 37°C. for 48 hours. The contents of all four tubes were then analyzed for amino, non-coagulable, and total nitrogen. Three experiments were carried out in this manner.

Non-coagulable nitrogen was determined as follows: 7 cc. of bacterial suspension were placed in a centrifuge tube and 7 cc. of 10 per cent trichloracetic acid were added. After standing for 1 hour the tube was centrifuged for 4 minutes at high speed. The supernatant liquid was filtered through quantitative filter paper. 6 cc. of the filtrate were analyzed for non-coagulable nitrogen by the Pregl micro Kjeldahl method.

The results of the analytical findings are given in Table III.

From the data presented in Table III it becomes evident that proteolysis is inhibited in solutions of pneumococci when the living cells are dissolved with an excess of sodium desoxycholate. The relation of this enzyme inhibition to the concentration of the bile salt in solutions of pneumococci is brought out in the following experiments.

3. Proteolysis in Solutions of Pneumococcus Containing Decreasing Amounts of Sodium Desoxycholate.

The preceding experiments showed that proteolysis is inhibited in solutions of pneumococci containing high concentrations of sodium

TABLE III.

Proteolysis in Sodium Desoxycholate Solutions of Pneumococcus (Type III) as Measured by the Increase in Amino and Non-Coagulable Nitrogen.

Exp. No.	Tube No.	pH	Total nitrogen	Non-coagulable nitrogen	Amino nitrogen	Nitrogen in amino form	Nitrogen in non-coagulable form	Increase in amino nitrogen	Increase in non-coagulable nitrogen
			mg. per cc.	*mg. per cc.*	*mg. per cc.*	*per cent*	*per cent*	*per cent*	*per cent*
A	1	6.5	0.69	0.29	0.107	15.5	42.1	—	—
	2	6.5	0.69	0.35	0.118	17.1	50.6	10.3	20.2
	3	6.5	0.67	0.28	0.103	15.4	41.8	0.0	—
	4	6.5	0.68	0.29	0.108	15.9	42.6	2.6	1.2
B	1	7.5	0.65	0.22	0.092	14.1	33.9	—	—
	2	7.5	0.65	0.32	0.109	16.8	49.3	19.2	45.5
	3	7.5	0.66	0.23	0.092	13.9	34.8	0.0	2.6
	4	7.5	0.66	0.22	0.092	13.9	33.3	0.0	—
C	1	7.5	0.57	0.23	0.099	17.4	40.4	—	—
	2	7.5	0.53	0.36	0.114	21.5	67.9	23.5	68.2
	3	7.5	0.56	0.23	0.095	17.0	41.1	0.0	1.7
	4	7.5	0.54	0.22	0.094	17.4	40.8	0.0	1.0

Tube 1. Control tube, 25 cc. of bacterial suspension. Heated 10 minutes at 100°C. to destroy active cellular enzymes.

Tube 2. 25 cc. of bacterial suspension to which was added 0.5 cc. of chloroform. Autolyzed 48 hours at 37°C.

Tube 3. 25 cc. of bacterial suspension; cooled to 0°C., then 1.5 cc. of 10 per cent desoxycholate added. After solution immediately heated at 100°C. for 10 minutes to destroy active cellular enzymes.

Tube 4. Same as Tube 3, but unheated. Autolyzed 48 hours at 37°C.

desoxycholate. In order to ascertain the minimum concentration of this reagent which would dissolve the cells and at the same time permit proteolysis to take place, experiments were carried out with decreasing concentrations of desoxycholate.

The bacteria from 3 liters of broth culture were collected as before. They were suspended in 100 cc. of isotonic salt solution, divided into four equal parts, cooled

TABLE IV.

Proteolysis in Sodium Desoxycholate Solutions of Pneumococcus (Type III) with Decreasing Concentrations of Desoxycholate, as Measured by the Increase in Amino and Non-Coagulable Nitrogen.

Exp. No.	Tube	pH	Total nitrogen	Non-coagulable nitrogen	Amino nitrogen	Nitrogen in amino form	Nitrogen in non-coagulable form	Increase in amino nitrogen	Increase in non-coagulable nitrogen	Remarks	Concentration of desoxycholate
			mg. per cc.	*mg. per cc.*	*mg. per cc.*	*per cent*	*per cent*	*per cent*	*per cent*		
G	1	NaCl	0.44	0.14	0.065	14.8	31.8	—	—	25 cc. suspension + 1 cc. 10 per cent sodium desoxycholate	1:250
	2	NaCl	0.43	0.14	0.064	14.9	32.5	0.7	2.2	25 cc. suspension + 0.5 cc. 10 per cent sodium desoxycholate	1:500
	3	NaCl	0.44	0.14	0.065	14.8	31.8	0.0	0.0	25 cc. suspension + 0.25 cc. 10 per cent sodium desoxycholate	1:1000
	4	NaCl	0.44	0.20	0.073	16.6	45.5	12.2	43.1	25 cc. suspension + 0.10 cc. 10 per cent sodium desoxycholate. All tubes incubated for 4 days at 37°C.	1:2500
H	1	NaCl	0.69	0.26	0.093	13.5	37.7	—	—	Same as experiment G	1:250
	2	NaCl	0.69	0.26	0.093	13.5	37.7	0.0	0.0		1:500
	3	NaCl	0.69	0.27	0.095	13.7	39.2	1.5	4.0		1:1000
	4	NaCl	0.69	0.35	0.114	16.5	50.8	22.2	34.8		1:2500

to 0°C., and to each part was added, respectively, 1.0, 0.5, 0.25, and 0.1 cc. of 10 per cent solution of sodium desoxycholate. The tubes were placed in the incubator at 37°C. for 48 hours. An analysis of the amino, non-coagulable, and total nitrogen was made, after heating for 10 minutes at 100°C. The analytical data are summarized in Table IV.

It is seen from the results of the experiments presented in Table IV that if the concentration of sodium desoxycholate in suspensions of pneumococci is diminished to 1 part in 2500, proteolysis progresses in the usual manner. It appears, therefore, that sodium desoxycholate in a concentration greater than 1:2500 inhibits the activity of the proteolytic enzyme of Pneumococcus.

4. Proteolysis Accompanying Autolysis of Pneumococcus under Aerobic and Anaerobic Conditions.

A comparison of proteolysis during autolysis under aerobic and anaerobic conditions is made in the following experiment.

For this purpose the washed bacteria were suspended in isotonic salt solution as in the preceding experiment. The bacterial suspension was divided into two equal portions. In order to have the bacteria in the control tube in soluble form similar to that of the autolysate, the former suspension was cooled to 0°C., and the bacteria dissolved by adding 2 cc. of 10 per cent solution of sodium desoxycholate. After complete solution of the organisms had taken place, the tube was immediately heated for 10 minutes at 100°C. to inactivate the cellular enzymes. 2 cc. of isotonic salt solution were added to the second portion, and the tube was sealed with 10 cc. of vaseline and incubated at 37°C. for 48 hours. The contents of both tubes were then analyzed for amino and non-coagulable nitrogen (Experiment 34).

A second experiment (Experiment 35) was carried out in a similar manner. 4 equal portions of a bacterial suspension were treated as follows: The first portion was cooled to 0°C. and 1 cc. of 10 per cent solution of sodium desoxycholate was added. After solution of the cells had taken place the tube was immediately heated for 10 minutes at 100°C. The second portion was also treated with desoxycholate but was not heated. This tube, containing the active enzymes, was incubated for 48 hours at 37°C. The third portion, consisting of the living formed cells, untreated with sodium desoxycholate was sealed with 10 cc. of vaseline and incubated at 37°C. for 48 hours. The fourth portion was treated precisely as the third except that it was incubated without vaseline seal. All four samples were then analyzed for amino and non-coagulable nitrogen. The results of both these experiments are summarized in Table V.

From the experiments presented in Table V it is seen that proteolysis progresses under anaerobic as well as under aerobic conditions and that, as pointed out in the preceding experiment (Table IV), solu-

TABLE V.

Proteolysis Accompanying Autolysis of Pneumococcus (Type III) under Aerobic and Anaerobic Conditions.

Exp. No.	Tube No.	pH	Total nitrogen	Non-coagulable nitrogen	Amino nitrogen	Nitrogen in amino form	Nitrogen in non-coagulable form	Increase in amino nitrogen	Increase in non-coagulable nitrogen
			mg. per cc.	*mg. per cc.*	*mg. per cc.*	*per cent*	*per cent*	*per cent*	*per cen*
34	1	NaCl	0.29	0.07	0.034	11.8	24.1	—	—
	2	NaCl	0.31	0.13	0.043	13.9	42.0	17.8	74.3
35	1	NaCl	0.235	0.055	0.022	9.4	23.4	—	—
	2	NaCl	0.235	0.055	0.023	9.8	23.4	4.3	0.0
	3	NaCl	0.235	0.075	0.027	11.5	31.9	22.3	36.3
	4	NaCl	0.235	0.09	0.027	11.5	38.3	22.3	63.8

Experiment 34.—Tube 1. The control tube: 35 cc. of bacterial suspension + 2 cc. of 10 per cent sodium desoxycholate at 0°C.; after solution heated immediately for 10 minutes at 100°C. to inactivate the enzymes.

Tube 2. The autolysate: 35 cc. of bacterial suspension, 2 cc. of saline, 1 cc. of chloroform, sealed with 10 cc. of sterile vaseline and incubated 48 hours at 37°C.

Experiment 35.—Tube 1. The control tube: 25 cc. of bacterial suspension, 1 cc. of 10 per cent desoxycholate at 0°C.; then after solution heated immediately for 10 minutes at 100°C.

Tube 2. Same as Tube 1, but unheated, and incubated for 48 hours at 37°C.

Tube 3. 25 cc. of bacterial suspension, active, no sodium desoxycholate added, sealed with 10 cc. of sterile vaseline, and incubated for 48 hours at 37°C.

Tube 4. 25 cc. of bacterial suspension, active, without seal, incubated 48 hours at 37°C.

tions of living bacterial cells made by dissolving the organisms with an *excess* of sodium desoxycholate, undergo no proteolysis.

5. Lipolysis in Pneumococcus Autolysates.

From the foregoing experiments it is evident that autolysis is accompanied by definite proteolysis. It is known that the Pneumo-

coccus contains an active lipase. It was thought that if this enzyme were active during the process of cell digestion, a demonstrable in-

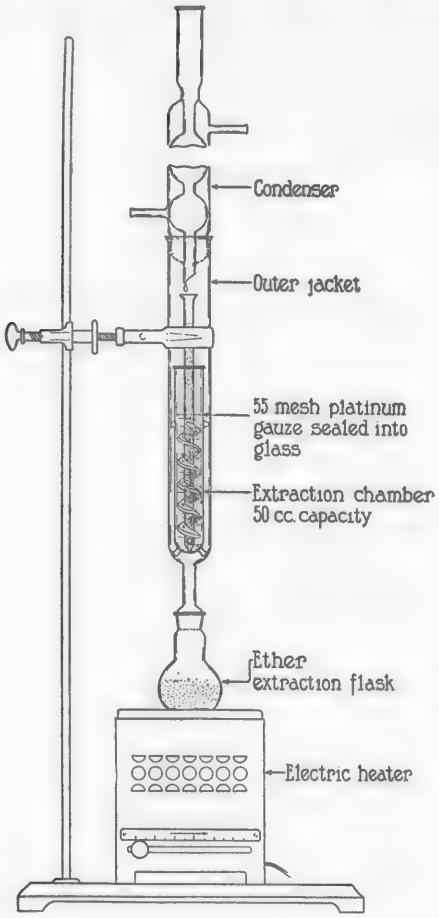

FIG. 1. Liquid extraction apparatus for estimating bacterial fatty acids.

crease in fatty acids should be found in pneumococcus autolysates when compared with the heated control of the same suspension, in which autolysis has been prevented by inactivation of the enzymes.

For the purpose of estimating fatty acids in bacterial suspensions and in autolysates, a small liquid extraction apparatus[1] was devised (Fig. 1).

A measured amount of bacterial substance was placed in the extraction chamber, 5 cc. of N/1 sulfuric acid were added, and the mixture was extracted for 24 hours, 75 cc. of purified ethyl ether[2] being used as a solvent. At the end of 24 hours extraction, the flask containing the ether and dissolved bacterial fatty acids was removed. The ether was evaporated over a warm water bath.

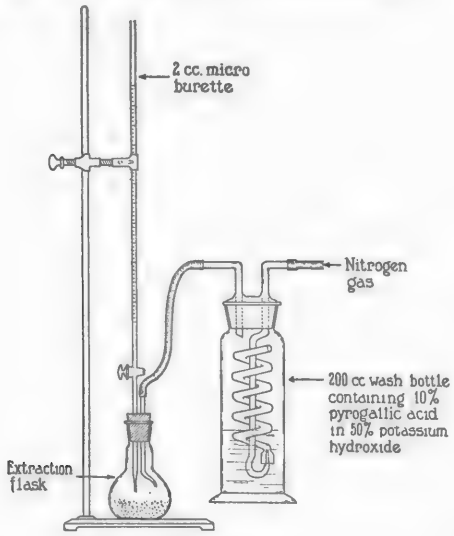
2 cc. micro burette
Nitrogen gas
200 cc wash bottle containing 10% pyrogallic acid in 50% potassium hydroxide
Extraction flask

FIG. 2. Apparatus for titrating bacterial fatty acids in an atmosphere of nitrogen.

The flask was now fitted with a 3-hole rubber stopper containing an intake tube for nitrogen gas, an opening for insertion of the micro burette, and a third opening

[1] The authors wish to express their thanks to Dr. P. A. Levene who suggested the principle of liquid extraction for the estimation of fatty acids.

[2] Ethyl ether was shaken in a separatory funnel with a saturated solution of calcium chloride to remove alcohol, then with alkaline potassium permanganate to remove aldehydes and acids, and finally with water. After drying over calcium chloride, the ether was twice distilled. 75 cc. of this ether, when evaporated to dryness, left a residue, which when dissolved in the purified alcohol described in Foot-note 2, neutralized 0.08 cc. N/100 sodium hydroxide.

for the introduction of the solvent. The flask was then filled with nitrogen gas which had been washed free from oxygen and carbon dioxide by bubbling through a 10 per cent solution of pyrogallic acid in 50 per cent potassium hydroxide. 5 cc. of purified ethyl alcohol[3] and 1 drop of 0.5 per cent phenolphthalein solution were introduced. The fatty acids in solution were then titrated with N/100 sodium hydroxide, by means of a special microcapillary burette of 2 cc. capacity, graduated in 1/100 cc. The first faint pink was taken as the end-point. Since

TABLE VI.

Lipolysis Accompanying Autolysis of Pneumococcus (Type III).

Exp. No.	Tube	pH	Bacterial nitrogen in sample extracted	N/100 NaOH to neutralize bacterial fatty acid	N/100 NaOH per mg. of bacterial nitrogen	Increase
			mg.	*cc.*	*cc.*	*per cent*
2	C*	NaCl	6.91	1.480	0.214	—
	A†	NaCl	5.70	1.580	0.276	28.9
3	C	NaCl	4.00	1.192	0.298	—
	A	NaCl	3.20	1.209	0.378	26.9
4	C	6.5	8.40	3.780	0.450	—
	A	6.5	7.60	6.740	0.887	97.2
5	C	6.5	10.00	3.181	0.318	—
	A	6.5	8.60	4.559	0.530	66.7
6‡	C	NaCl	—	2.134	—	—
	A	NaCl	—	3.461	—	62.2

* C = control tube.

† A = autolysate.

‡ Alcoholic extract of pneumococci, suspended in salt solution, and to which had been added pneumococcus extract containing active enzymes (Tube A) and heat-inactivated enzymes (Tube C).

phenolphthalein is sensitive to carbon dioxide, the titration was carried out in an atmosphere of nitrogen. In this manner a most accurate titration and lasting end-point were obtained. Three blank determinations, with 2.84 mg. of crystalline stearic acid, gave titrations of 0.990, 0.995, and 1.000 cc. N/100 sodium hydroxide, after subtracting a blank of 0.08 cc. N/100 sodium hydroxide for the ether and alcohol. A diagram of the titration apparatus is given in Fig. 2.

The results of four experiments are given in Table VI.

[3] Absolute ethyl alcohol was twice distilled over sticks of potassium hydroxide, and then once without potassium hydroxide.

6. Lipolysis in Solutions of Pneumococcus Containing Sodium Desoxycholate.

It has been pointed out above that sodium desoxycholate inhibits the action of the proteolytic enzyme of Pneumococcus. In order to ascertain whether the lipase is still active in solutions of Pneumococcus containing sodium desoxycholate, the following experiments were undertaken.

The growth from 3 liters of broth culture of Type III pneumococcus was obtained as in previous experiments. The living bacteria were taken up in 50 cc.

TABLE VII.

Lipolysis in Sodium Desoxycholate Solutions of Pneumococcus (Type III).

Exp. No.	Tube	pH	Bacterial nitrogen in sample extracted	N/100 NaOH to neutralize the extract	Increment of increase in terms of cc. of N/100 NaOH
			mg.	*cc.*	
F	C*	7.5	12.8	12.13	—
	A†		12.8	14.60	2.47
H	C	7.5	7.32	14.30	—
	A		7.32	16.00	1.70
I	C	7.5	7.30	11.95	—
	A		7.30	14.11	2.16
J	C	7.5	7.32	11.85	—
	A		7.32	13.67	1.82

* C = control tube.
† A = autolysate.

of M/15 phosphate buffer at pH 7.5, the suspension was cooled in ice to 0°C., and to it were added 2 cc. of a 10 per cent solution of sodium desoxycholate. After complete solution of the bacterial cells had occurred in the cold, 25 cc. of the mucoid-like material were inactivated by heating for 10 minutes at 100°C. as control; and the remaining 25 cc. were allowed to autolyze at 37°C. for 48 hours. 0.5 cc. of chloroform was added as a preservative.

After incubation, and subsequent evaporation of the chloroform, 20 cc. of these solutions were placed in the liquid extraction apparatus and each extracted for 24 hours as described above. The extracts were then titrated.

The results of these experiments are given in Table VII.

In order to determine whether lipolysis accompanies the solution of pneumococci by bile at 0°C., the following experiments were carried out.

The growth from 3 liters of Pneumococcus Type III was obtained as in 1. The bacteria were suspended in 60 cc. of isotonic salt solution. Two portions of 25 cc. each of living organisms were quantitatively measured into two tubes. The first tube, as control, was immediately heated for 10 minutes at 100°C., then cooled, and to the suspension of heat-killed bacteria exactly 2 cc. of a 10 per cent solution of sodium desoxycholate were added. The second tube, unheated, was kept at 0°C. and to it were added exactly 2 cc. of the same solution of desoxycholate.

TABLE VIII.

The Absence of Lipolysis during Solution of Pneumococci (Type III) by Sodium Desoxycholate at 0°C.

Exp. No.	Tube	pH	Bacterial nitrogen in sample extracted	N/100 NaOH to neutralize the extract	Increment of increase in terms of cc. of N/100 NaOH
			mg.	*cc.*	
1	a*	NaCl	6.32	20.12	—
	b†	NaCl	6.32	20.14	0.02
2	a	NaCl	5.93	18.63	—
	b	NaCl	5.91	18.64	0.01

* a = Heated control.

† b = Sodium desoxycholate solutions of living pneumococci.

After complete solution of the organisms in the cold, this tube was plunged directly into boiling water for 10 minutes to inactivate the enzymes.

20 cc. of each of the two samples were placed in the liquid extractor, extracted for 24 hours, and the extract titrated with N/100 sodium hydroxide.

The results are given in Table VIII.

In Tables VI to VIII are found the results of experiments on the occurrence of lipolysis during pneumococcus autolysis. It is seen that the lipase is active not only during normal autolysis, as measured by the increase in ether-soluble fatty acids, but that after solution of the cells by sodium desoxycholate, it retains its activity even in the presence of relatively high concentrations of the bile salt. When, however, pneumococci are dissolved at 0°C. with sodium desoxycholate,

and the solution immediately heated, no increase in fatty acid is found to have occurred. In other words, the mere solution of pneumococci by sodium desoxycholate at 0°C., a temperature at which enzyme action is retarded, is not accompanied by lipolysis.

DISCUSSION.

The phenomenon of pneumococcus autolysis is accompanied by two distinct chemical changes: first, enzymatic degradation of some protein or proteins of the cell into less complex molecules; second, a liberation of free fatty acids, probably of high molecular weight, by the action of pneumococcus lipase on certain cellular esters. On the other hand, when bile (sodium desoxycholate) solutions of pneumococci are incubated, only lipolysis and no proteolysis takes place when an excess of the bile salt is used.

Throughout the literature there are statements to the effect that the solution of pneumococci in bile is due to an acceleration of normal autolytic processes; in the present study it has been found that proteolysis does not occur when pneumococci are dissolved in the presence of an excess of sodium desoxycholate. Furthermore, pneumococci can be brought rapidly into solution by sodium desoxycholate at temperatures (0°C.) where the rate of enzyme action is retarded, and where one would scarcely expect either proteolysis, lipolysis, or any other enzyme action to play an important rôle. Under these conditions it seems difficult, therefore, to conceive of the solution of pneumococci by bile as an acceleration of the normal autolytic process, since the chemical changes which accompany *true autolysis* are not associated with *solution* of the organisms by bile.

Julianelle and Reimann (11) have shown that extracts of pneumococci contain a toxic principle capable of producing experimental purpura in animals. They pointed out that bile solutions of pneumococci do not yield the purpura-producing substance. They concluded that: "it appears probable, therefore, that the purpura-producing property is due not to a constituent of the living pneumococcus cell, but to a degradation product of pneumococcus." Mair (12), on the other hand, has found that bile and sodium desoxycholate solutions of pneumococci produce purpura in mice. He attributes the differences between his results and those of Julianelle and Reimann to differences

in technique. The analytical data in the present investigation show at least that proteolysis does not accompany solution of pneumococci, if the concentration of sodium desoxycholate is greater than 1 part in 2500.

It has been pointed out that in autolysates and in bile solutions of pneumococci, the type-specific antigen undergoes a change whereby it loses in greater part the capacity to stimulate type-specific antibodies. On the other hand, the cell proteins in soluble form retain their antigenic properties and induce only the species-specific antibodies; that is, the dissolved substances yield a serum containing a high titer of antiprotein antibodies. The immunological evidence, therefore, supports the view that during autolysis and dissolution of the cell, particularly under conditions which release the intracellular enzymes in an active state, the complex antigen is broken down into its component parts, one of which, at least, consists of the antigenically inert hapten,—the type-specific carbohydrate. While the existing information emphasizes the importance of the principle of antigenic dissociation, it is, as yet, too meager to admit of any decision as to the nature of the chemical union or the factors which determine the rate and extent of dissociation of the conjoined antigen in the test-tube and in the animal body.

Although earlier investigators have isolated carbohydrate material in the form of bacterial gums from a variety of microorganisms, the relation of these polysaccharides to the immunological specificity and antigenic properties of the cell has only recently been recognized. While reasoning by analogy is often fallacious, the relation of the carbohydrate radicle to the conjugated protein of "animal gums" is at least suggestive that a similar relationship may exist in the carbohydrate-protein complex of Pneumococcus. In the group of conjugated proteins of animal origin, Levene (13) has pointed out that the carbohydrate radicle is bound to the protein by a quite simple and comparatively labile union in the nature of an ester linkage. If similar relations prove to exist in the case of Pneumococcus, an antigenic linkage of this nature would be susceptible to most chemical agents and to action of the cell's own esterase, which, as the present study shows, is active during autolysis and in the presence of bile salts. Under these conditions, the differences observed in the

rate and degree of antigenic dissociation of the three fixed types of Pneumococcus would be referable to the particular form of linkage, dependent upon known differences in the chemical structure of the type-specific polysaccharides. For example, the complex sugar of Type I contains nitrogen partly in amino form, while the corresponding carbohydrates of both Type II and Type III are each nitrogen-free. From the chemical point of view, therefore, the greater stability of the Type I antigen might be conceived of as due to a more resistant linkage or one of a different order than that found in the other two types. Similarly, from an immunological point of view, the most efficient of these complex antigens would be the one least easily dissociable; that is, in terms of type specificity, the antigenic potency of any given type of Pneumococcus would be inversely proportional to the rate and extent of antigenic dissociation.

The results of the present study of autolysis, as well as the earlier observations on the differences in antibody response of animals to the different types of Pneumococcus, suggest the possible relationship of enzyme action to antigenic dissociation *in vitro* and *in vivo*.

CONCLUSIONS.

1. Autolysis of Pneumococcus is accompanied by proteolysis, which results in an increase in amino and non-coagulable nitrogen.

2. Autolysis of Pneumococcus is accompanied by lipolysis during which there is a liberation of ether-soluble fatty acids.

3. When extracts containing the active intracellular enzymes are added to heat-killed pneumococci, lysis of the cells occurs and there is an increase in the non-coagulable and amino nitrogen, comparable to the changes accompanying spontaneous autolysis.

4. When extracts containing the active intracellular enzymes are added to emulsions of the alcohol-soluble lipoids extracted from pneumococci, an increase in the ether-soluble fatty acid occurs.

5. Sodium desoxycholate in excess inhibits the action of pneumococcus protease; it does not inhibit the action of pneumococcus lipase.

6. When suspensions of pneumococci are cooled to 0°C., a temperature at which the rate of enzyme action is greatly retarded, the organisms go into solution rapidly when sodium desoxycholate is added, but this process is not accompanied by lipolysis or proteolysis. It

does not seem probable, therefore, that the "bile" solution of pneumococci is identical with the phenomenon of autolysis as ordinarily understood and measured.

7. The relation of enzyme action to antigenic dissociation is discussed.

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BIOMETRY OF CALCIUM, INORGANIC PHOSPHORUS, CHOLESTEROL, AND LIPOID PHOSPHORUS IN THE BLOOD OF RABBITS.

II. Repeated Observations on Normal Animals.

By ALVIN R. HARNES, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, October 22, 1928.)

The results obtained from a series of determinations of calcium, inorganic phosphorus, cholesterol, and lipoid phosphorus on normal animals from recently acquired stock were reported in a previous paper (1). These results were based on single determinations made on animals within a few days after they were received from the dealer. A similar series of repeated blood analyses was made on a group of 10 animals living in the laboratory for the purpose of determining whether the calcium, inorganic phosphorus, cholesterol, and lipoid phosphorus differed in any way from that of animals living out in the open. The results obtained for animals living in the laboratory will be reported in the present paper and compared with those reported for animals living out-of-doors.

Materials and Method.

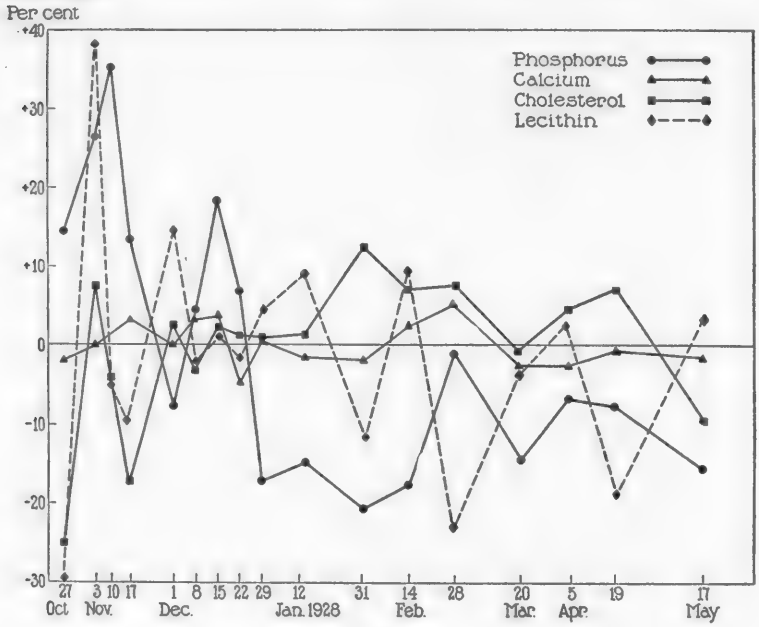
The data presented in this paper are derived from experiments, the procedures of which were described in detail in a preceding paper (1).*

In the experiment 10 male rabbits between 6 and 8 months of age were used. This group was composed of 4 greys, 4 Flemish, and 2 mixed grey-brown animals. From the beginning of the experiment on October 27, 1927, until May 17, 1928, these animals were kept in the open laboratory, caged separately, and fed the routine diet of hay, oats, and cabbage.

* In the determination of lipoid phosphorus as published originally (Harnes, A. R., *J. Biol. Chem.*, 1928, lxxvii, 405), the H_2SO_4 used in making all reagents should have been arsenic-free.

The determinations of calcium and inorganic phosphorus on blood serum and cholesterol and lipoid phosphorus on whole blood were made on the same sample of blood. Determinations were made at weekly or biweekly intervals resulting in 17 examinations for the entire period of the experiment.

In all text-figures and tables the lipoid phosphorus is calculated and presented as lecithin.

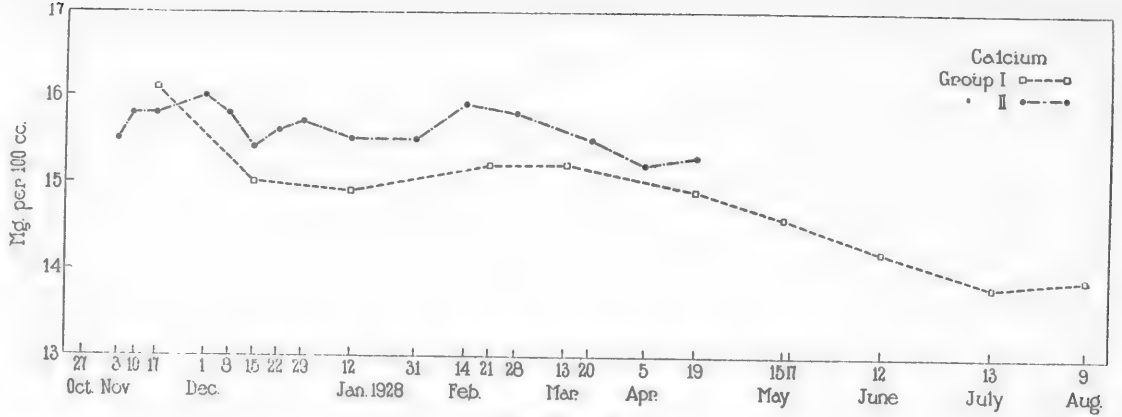


TEXT-FIG. 1.

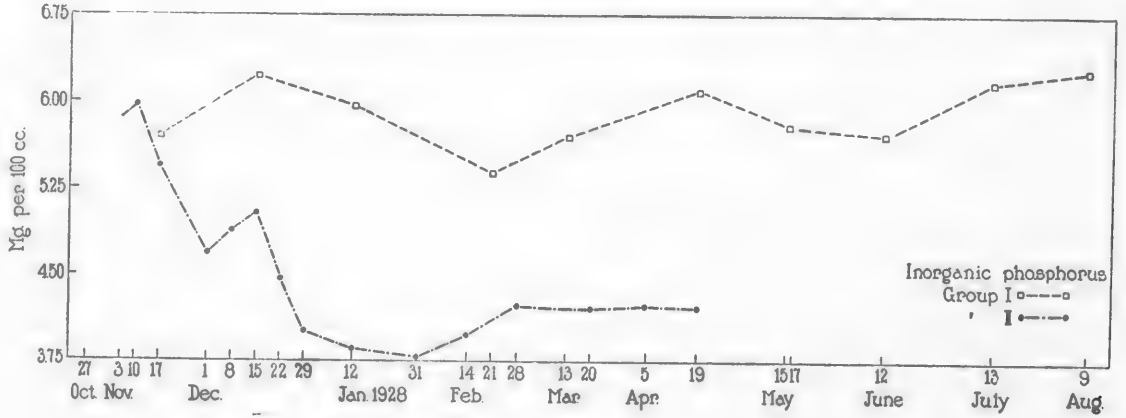
RESULTS.

The results of this experiment are reported in the form of tabulated summaries, Tables I to III, and supplemented by a series of graphs, Text-figs. 1 to 5. The curves in Text-figs. 2 to 5 inclusive represent values that have been smoothed by the formula $\frac{A + 2B + C}{4}$.

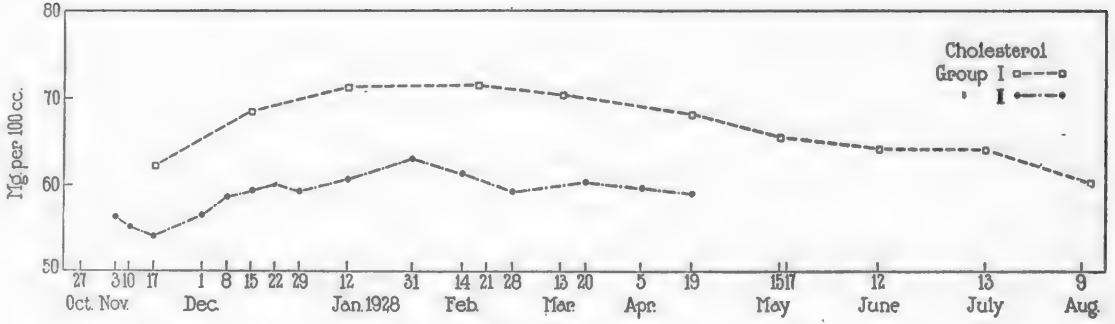
The per cent variation from the mean or average value for each of the 4 constituents tabulated in Table I is presented graphically in Text-fig. 1. The values for calcium and inorganic phosphorus are



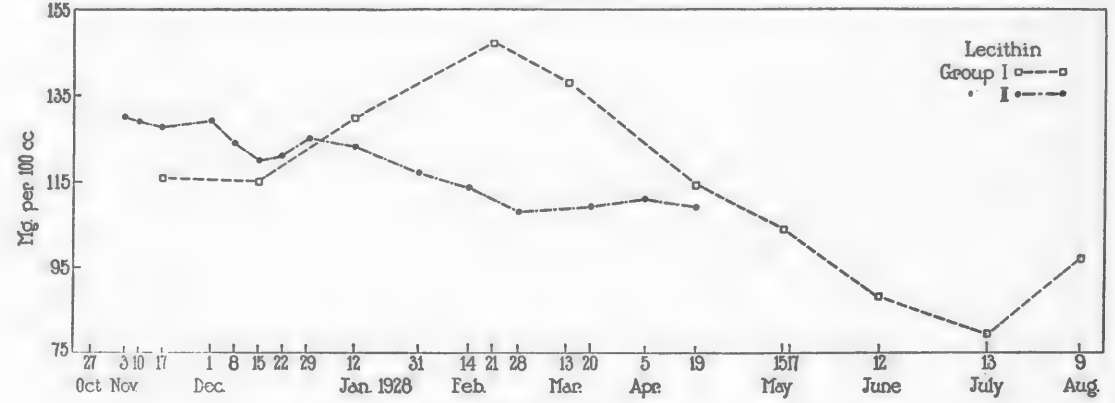
TEXT-FIG. 2.


Inorganic phosphorus
Group I
I
Mg. per 100 cc.
6.75
6.00
5.25
4.50
3.75
27 3 10 17 1 8 15 22 29 12 31 14 21 28 13 20 5 19 15 17 12 13 9
Oct. Nov. Dec. Jan. 1928 Feb. Mar Apr May June July Aug.

TEXT-FIG. 3.


Cholesterol
Group I
I
Mg. per 100 cc.
80
70
60
50
27 3 10 17 1 8 15 22 29 12 31 14 21 28 13 20 5 19 15 17 12 13 9
Oct. Nov. Dec. Jan. 1928 Feb. Mar. Apr. May June July Aug.

TEXT-FIG. 4.



TEXT-FIG. 5.

TABLE I.

Group Mean Values for Consecutive Determinations.

Date	Calcium			Inorganic phosphorus			Cholesterol			Lecithin		
	Mean	Standard deviation	Coefficient of variation	Mean	Standard deviation	Coefficient of variation	Mean	Standard deviation	Coefficient of variation	Mean	Standard deviation	Coefficient of variation
1927	*mg. per 100 cc.*	*mg.*	*per cent*	*mg. per 100 cc.*	*mg.*	*per cent*	*mg. per 100 cc.*	*mg.*	*per cent*	*mg. per 100 cc.*	*mg.*	*per cent*
Oct. 27	15.3 ±0.288	1.35	8.80	5.348 ±0.109	0.51	9.54	43.5 ±1.48	6.95	15.95	82.9 ±2.68	12.55	15.40
Nov. 3	15.6 ±0.352	1.65	10.56	5.888 ±0.175	0.82	13.93	62.6 ±1.27	5.95	9.52	164.1 ±3.27	17.40	10.60
" 10	16.1 ±0.144	0.68	4.19	6.290 ±0.162	0.76	12.08	55.8 ±0.76	3.55	6.14	112.6 ±1.31	6.16	5.47
" 17	15.6 ±0.093	0.44	2.81	5.295 ±0.095	0.45	8.41	48.3 ±1.63	7.63	15.79	129.4 ±3.26	15.30	11.83
Dec. 1	16.1 ±0.149	0.70	4.35	4.290 ±0.075	0.35	8.23	59.7 ±1.18	5.55	9.30	136.0 ±1.49	6.97	5.12
" 8	16.2 ±0.179	0.84	5.18	4.868 ±0.100	0.47	9.65	56.1 ±1.39	6.51	11.60	115.2 ±2.63	12.34	10.71
" 15	14.9 ±0.147	0.69	4.62	5.514 ±0.096	0.45	8.16	59.9 ±1.38	6.45	10.76	120.7 ±2.31	10.84	8.98
" 22	15.7 ±0.165	0.77	4.91	4.980 ±0.331	1.55	35.96	61.2 ±1.27	5.97	9.75	116.4 ±2.34	11.00	9.45
" 29	16.2 ±0.200	0.94	5.79	3.860 ±0.069	0.32	8.47	58.6 ±0.82	3.82	6.51	123.5 ±2.91	13.62	11.02
1928												
Jan. 12	15.4 ±0.146	0.68	4.44	3.966 ±0.057	0.27	6.78	59.2 ±0.74	3.48	5.88	129.1 ±2.73	12.81	9.92
" 31	15.3 ±0.155	0.73	4.73	3.688 ±0.074	0.35	9.38	65.6 ±1.57	7.36	11.21	104.8 ±1.52	7.11	6.78
Feb. 14	16.0 ±0.410	1.93	12.05	3.820 ±0.076	0.36	9.37	62.3 ±0.82	3.84	6.16	129.7 ±2.11	9.90	7.63
" 28	16.4 ±0.113	0.53	3.23	4.607 ±0.083	0.39	8.40	62.8 ±2.86	13.40	21.34	94.3 ±2.79	13.09	13.88
Mar. 20	15.2 ±0.113	0.53	3.48	3.990 ±0.088	0.41	10.32	57.9 ±1.12	5.23	9.03	114.1 ±5.16	24.20	21.20
Apr. 5	15.2 ±0.126	0.59	3.90	4.339 ±0.208	0.98	22.49	60.8 ±0.63	2.95	4.85	121.3 ±3.15	14.79	12.19
" 19	15.5 ±0.118	0.55	3.57	4.303 ±0.127	0.59	13.80	62.3 ±1.06	4.96	7.95	96.7 ±2.34	10.98	11.35
May 17	15.3 ±0.144	0.67	4.41	3.933 ±0.055	0.26	6.51	52.7 ±1.11	5.22	9.91	122.4 ±1.51	7.08	5.78

TABLE II.

Coefficients of Correlation for Consecutive Group Determinations.

Date	rP.Ca. *P.E.*	rP.Chol. *P.E.*	rP.Lec. *P.E.*	rCa.Chol. *P.E.*	rCa.Lec. *P.E.*	rChol.Lec. *P.E.*
1927						
Oct. 27	+0.086 ±0.212	−0.335 ±0.191	−0.217 ±0.203	+0.067 ±0.213	−0.427 ±0.174	−0.119 ±0.210
Nov. 3	−0.169 ±0.207	+0.087 ±0.212	−0.538 ±0.152	−0.038 ±0.213	−0.367 ±0.185	+0.193 ±0.205
" 10	−0.324 ±0.191	−0.511 ±0.158	+0.231 ±0.202	−0.331 ±0.190	+0.337 ±0.189	+0.082 ±0.212
" 17	−0.035 ±0.213	+0.172 ±0.207	+0.180 ±0.206	+0.471 ±0.166	−0.016 ±0.213	−0.222 ±0.202
Dec. 1	−0.176 ±0.207	−0.308 ±0.193	+0.341 ±0.188	+0.086 ±0.212	+0.577 ±0.142	−0.415 ±0.177
" 8	−0.210 ±0.204	−0.218 ±0.204	−0.071 ±0.212	+0.068 ±0.213	−0.044 ±0.213	−0.123 ±0.210
" 15	−0.266 ±0.198	+0.275 ±0.197	−0.035 ±0.213	+0.212 ±0.203	+0.243 ±0.201	+0.276 ±0.197
" 22	−0.827 ±0.067	−0.511 ±0.158	+0.222 ±0.203	+0.047 ±0.213	−0.159 ±0.208	−0.248 ±0.200
" 27	−0.226 ±0.202	+0.628 ±0.129	−0.508 ±0.158	−0.001 ±0.213	+0.640 ±0.126	−0.133 ±0.210
1928						
Jan. 12	−0.103 ±0.211	−0.068 ±0.212	+0.501 ±0.160	+0.427 ±0.174	−0.311 ±0.193	+0.412 ±0.177
" 31	−0.271 ±0.198	+0.354 ±0.187	−0.161 ±0.208	+0.564 ±0.145	+0.077 ±0.212	+0.194 ±0.205
Feb. 14	−0.281 ±0.198	+0.168 ±0.207	+0.090 ±0.212	−0.252 ±0.200	−0.741 ±0.096	+0.458 ±0.169
" 28	−0.031 ±0.213	+0.078 ±0.212	+0.171 ±0.207	−0.124 ±0.210	+0.182 ±0.206	−0.136 ±0.210
Mar. 20	−0.034 ±0.213	+0.537 ±0.152	+0.537 ±0.152	+0.328 ±0.190	−0.561 ±0.146	+0.392 ±0.181
Apr. 5	−0.423 ±0.175	+0.075 ±0.212	−0.288 ±0.196	−0.096 ±0.213	+0.218 ±0.203	−0.476 ±0.165
" 19	+0.172 ±0.207	−0.226 ±0.202	−0.100 ±0.211	−0.106 ±0.211	+0.423 ±0.174	−0.074 ±0.212
May 17	−0.089 ±0.212	−0.106 ±0.211	−0.093 ±0.213	+0.516 ±0.158	−0.397 ±0.180	−0.670 ±0.117

TABLE III.

Section (a). Coefficients of Correlation of Group Means.

rP.Ca. *P.E.*	rP.Chol. *P.E.*	rP.Lec. *P.E.*	rCa.Chol. *P.E.*	rCa.Lec. *P.E.*	rChol.Lec *P.E.*
$+0.027 \pm 0.163$	-0.327 ± 0.146	$+0.103 \pm 0.162$	$+0.153 \pm 0.160$	$+0.002 \pm 0.163$	$+0.240 \pm 0.154$

Section (b). Coefficients of Correlation of 170 Individual Values.

rP.Ca. *P.E.*	rP.Chol. *P.E.*	rP.Lec. *P.E.*	rCa.Chol. *P.E.*	rCa.Lec. *P.E.*	rChol.Lec. *P.E.*
-0.124 ± 0.051	-0.147 ± 0.051	$+0.087 \pm 0.051$	$+0.078 \pm 0.051$	-0.036 ± 0.051	$+0.138 \pm 0.051$

Section (c). Coefficients of Correlation for Individual Mean Values.

rP.Ca. *P.E.*	rP.Chol. *P.E.*	rP.Lec. *P.E.*	rCa.Chol. *P.E.*	rCa.Lec. *P.E.*	rChol.Lec. *P.E.*
-0.864 ± 0.047	$+0.481 \pm 0.129$	-0.164 ± 0.150	-0.041 ± 0.171	-0.029 ± 0.191	$+0.391 \pm 0.130$

given in mg. per 100 cc. of blood serum, while the results for cholesterol and lecithin are given in mg. per 100 cc. of whole blood. In Text-figs. 2 to 5 inclusive a comparison is made between Group I which shows the results presented in a previous paper (1) and is more or less representative of animals living out in the open, and Group II results obtained from animals living in the laboratory.

DISCUSSION AND CONCLUSION.

Variation.

Calcium.—It will be noted that the trend of the curves for calcium (Text-fig. 2) is essentially the same in both groups of animals. The animals living in the laboratory maintained a higher level of calcium than those just received from the dealer. Both curves show a drop in calcium during December and January and an increase during February and March. With the animals in Group I there is a gradual decrease in calcium throughout the early spring and summer months, while with Group II there is a slight suggestion of an increase at the time of the last observation. The highest values were obtained in December and February and the lowest during December and April.

The results obtained for calcium show the least degree of variation of the substances studied, either in mg. per 100 cc. or in per cent variation from the mean. From the beginning of the experiment on Oct. 27, 1927, until Dec. 8, the values obtained for calcium increased from 15.3 $\pm$0.28 to 16.2 $\pm$0.17 mg. per 100 cc. of serum. On Dec. 15 the amount of calcium was found to be 14.9 $\pm$0.14, this being the smallest amount of calcium found throughout the experiment. From Dec. 29 to May 17, 1928, the amount of calcium varied less than 1 mg. per 100 cc. of serum from its mean value of 15.7 $\pm$0.05. The greatest degree of variation and also the most abrupt changes in calcium content of serum occurred during the first 6 weeks of the experiment.

Inorganic Phosphorus.—In considering the trend of inorganic phosphorus, Text-fig. 3, as compared with animals living in the open, a striking difference is noted. In Group II there was first a slight increase followed at once by a decrease in inorganic phosphorus which continued over a period of 4 months, from October to February, with the exception of a slight increase in November and December. During

the last half of February there was an increase in phosphorus from its minimum level with more or less stabilization which continued through the months of March, April, and May. Group I throughout this same period of time maintained a very much higher level of inorganic phosphorus and at no time during the entire year did it reach a level as low as that of Group II. The highest values for inorganic phosphorus occurred during November, the lowest in January.

In mg. of the element and per cent variation from its mean, phosphorus proved to be one of the most variable constituents. Beginning on Oct. 27, 1927, with a value of 5.34 ±0.10, the phosphorus after 2 weeks time was found to be 6.29 ±0.16 mg. per 100 cc. of serum. The following 2 weeks the phosphorus decreased rather abruptly and on Dec. 1 was found to be 4.29 ±0.07 mg. On Dec. 15 another increase in phosphorus was noted although not nearly as great as the value obtained following the first 2 weeks of the experiment. It was not until Dec. 29 that the phosphorus reached any degree of stability. On this date the amount in the blood serum was found to be 3.86 ±0.06 mg. per 100 cc. It may also be noted that in Text-fig. 1 the greatest per cent variation of phosphorus occurred during this first 2 months period. Except for the value of 4.60 ±0.08 obtained on Feb. 28, the changes in the phosphorus content of blood serum were very small. The mean value for phosphorus throughout the experiment was calculated to be 4.65 ±0.05 mg. per 100 cc. of blood serum.

The results for inorganic phosphorus agree in all essential respects with those obtained by Brown (2, 3) for animals living under the same conditions. The general averages for calcium are also in agreement as is shown by the following comparison of results:

	Combined results of Brown	Author's results
	mg. per 100 cc.	*mg. per 100 cc.*
Calcium	15.6 ±0.03	15.7 ±0.05
Inorganic phosphorus	4.51 ±0.02	4.65 ±0.05

There was, however, a difference in the trend of the curves for calcium. In these experiments higher values were obtained during the 1st month or 6 weeks, and the reduction in calcium which followed persisted with some variation until the end of January. In both cases, however, a high level was obtained either in January or February.

A comparison of the mean values obtained in this experiment was made with the combined mean values of last year because, while Group I of last year represents in time more nearly this year's experiment, it was made up of animals of distinctly different breeds and it seemed desirable to eliminate any difference which might be attributable to this factor. It was for this reason that the combined mean value of all animals of last year was used.

Cholesterol.—A comparison of the trend of cholesterol between Groups I and II is shown in Text-fig. 4. The outstanding features of these curves are two: first, animals living in the laboratory show the same progressive increase in cholesterol during the winter months with a decrease occurring during the early spring; and second, the general level of cholesterol for animals living in the laboratory is very much lower than was found to exist in animals living in the open. Since both calcium and cholesterol maintained similar trends in the 2 groups of animals, these facts give added significance to the high coefficient of correlation obtained between these 2 blood constituents for animals living out in the open. The periods of lowest values of cholesterol occurred during November and April while the high values occurred during January and February.

The stability of cholesterol was very marked throughout the entire experiment. Beginning on Oct. 27 with a value of 43.5 $\pm$1.48 the cholesterol increased to 62.6 $\pm$1.27 mg. per 100 cc. of whole blood on Nov. 3. During the next 2 weeks there was a gradual drop, and on Nov. 17 the value obtained was 48.3 $\pm$1.63 mg. The following examination made on Dec. 1 showed an increase in cholesterol, the amount being 59.7 $\pm$1.18 mg. per 100 cc. of whole blood. The remaining values obtained for cholesterol show only gradual changes from the mean which over the period of 8 months was found to be 58.3 $\pm$0.39 mg. per 100 cc. of whole blood.

Lecithin.—The trend of the curves for lecithin in animals living indoors is in a direction which is practically the opposite of that found for animals living out-of-doors (Text-fig. 5). With animals living in the laboratory the lecithin decreased in amounts from the very beginning of the experiment. While Group II maintained a higher level than Group I for the first 2 months of the experiment, Group I throughout January and until May showed an increase in lecithin,

while Group II steadily decreased. In comparing the trends of inorganic phosphorus and lecithin in these 2 groups, it will be noted that animals living in the laboratory show a marked decrease in both of these blood constituents when compared with animals living out in the open. The highest values for lecithin were found during November, the lowest values occurred during February and April.

Lecithin began on Oct. 27 with a value of 82.9 ±2.68 mg. per 100 cc. of whole blood. The next determination gave a result of 164.1 ±3.27 mg., this being the highest value for lecithin throughout the entire experiment. From this high value the lecithin decreased to 112.6 ±1.31 mg. on Nov. 10. It was not until the next determination on Nov. 17 of 129.4 ±3.26 mg. that lecithin reached a certain degree of stability. When compared with the other 3 constituents, lecithin showed the highest per cent variation and the least degree of stability throughout the entire experiment.

The variation of each constituent in terms of per cent from their respective mean values is given in Text-fig. 1. It will be noted that with animals living in the laboratory the greatest variation occurred within the first 6 weeks of the experiment. During this time the per cent variation for calcium was from −1.9 to +3.9, for inorganic phosphorus from +35.2 to −17.0, for cholesterol from −25.2 to +7.5, and for lecithin from −29.9 to +38.6. The occurrence of such wide variations during this period, when compared with the extent of subsequent variations, indicates that the results for the first 6 weeks are more or less affected by adjustment of the animals to a change of environmental conditions or mode of life.

The following table summarizes the mean values for the 2 groups of animals over the same period of time, from Oct. 27, 1927, to May 17, 1928. Group I represents the mean value of animals just received from the dealers and Group II the mean value of animals living under laboratory environment.

Group	Inorganic phosphorus	Calcium	Cholesterol	Lecithin
	mg. per 100 cc.	*mg. per 100 cc.*	*mg. per 100 cc.*	*mg. per 100 cc.*
I	5.8 ±0.08	15.5 ±0.14	66.8 ±0.84	124.5 ±2.47
II	4.6 ±0.05	15.7 ±0.05	58.2 ±0.39	118.4 ±1.13

It will be noted that the mean value for inorganic phosphorus, cholesterol, and lecithin is lower for animals living in the laboratory than was found to exist in animals just received from the dealer.

The results have been analyzed from the point of view of the distribution of values, but as it was found that in this respect the 2 groups of animals were almost identical, discussion of these results is omitted.

Correlation.

The coefficient of correlation for the 6 possible combinations of inorganic phosphorus, calcium, cholesterol, and lecithin on the 10 animals at each of the 17 determinations is given in Table II. Table III, Section (*a*), gives the coefficient of correlation for the total 170 individual determinations; Section (*b*) gives the coefficient of correlation on group mean values and, therefore, represents the correlation of the trend of the curves presented in Text-figs. 2 to 5 inclusive; Section (*c*) gives the coefficient of correlation of mean values obtained for each animal throughout the experiment. The same formula for calculating the coefficient of correlation was used as presented in a previous paper (1).

The coefficient obtained by using the mean value for individual animals gives the highest degree of correlation and more nearly resembles the correlation of trend for animals living out-of-doors. In calculating the mean value of calcium, inorganic phosphorus, cholesterol, and lecithin for the individual animals, it was noted that different animals tend to maintain or operate on different levels. Certain animals maintain throughout the year a high value for calcium, while in others this value is comparatively low. It was also noted that in calculating the coefficient of correlation between these 4 constituents on group means, 1 constituent would lag or anticipate the corresponding change in the other, thus the resulting coefficient would be comparatively small. This fact is brought out in Tables II and III, Sections (*a*) and (*b*). If, however, the average for each animal is obtained and a correlation coefficient calculated from these mean values, the factor of lag in one or the other constituent is eliminated and the resulting coefficient represents the relationship existing in the animal over the period of the 17 determinations.

In Table II only 1 series of correlations, rP.Ca., offers any degree of

uniformity. In this series the coefficient of correlation is negative except for the determinations made on Oct. 17, 1927, and April 19, 1928. It will be noted, however, that while the coefficients for each determination were negative, the *P.E.* in all instances except one is too large for the coefficients to have any degree of mathematical significance.

The coefficients of correlation presented in Table III, Sections (*a*) and (*b*), while too small for mathematical consideration, do serve to show that there is a marked difference in these 4 blood constituents between animals living out in the open and those living in the laboratory. With the animals just received from the dealer, there is a well defined and related trend of calcium, inorganic phosphorus, cholesterol, and lecithin, while with the animals living in the laboratory there is first a wide and abrupt variation covering a period of about 6 weeks and then a period of regularity. The coefficients of correlation for individual group determinations, the trend of the various curves, or the entire 170 single analyses show no factor of correlation of mathematical significance.

The results of this experiment show that when animals are brought into the laboratory from out-of-doors, they pass through a period of adjustment or accommodation. In this experiment the period of adjustment covered about 6 weeks. In addition to the period of adjustment there is a marked difference in both absolute values and trend for calcium and inorganic phosphorus in the blood serum and cholesterol and lecithin of whole blood when compared to the values found in animals just received from the dealer. It was found that while the trend of calcium for animals living in the laboratory was the same as that found in animals living in the open, the general level over the same period of time was higher for the animals living in the laboratory. The inorganic phosphorus showed a marked decrease in the animals living in the laboratory, while with animals out-of-doors a higher level with increasing values was obtained. The trend of the curves for cholesterol of whole blood was the same for both groups of animals; however, the animals living in the laboratory maintained throughout the experiment a decidedly lower level than was found to exist in the blood of animals living out-of-doors. The lecithin which gave a higher value at the beginning of the experiment than was found

in animals living out-of-doors continued to decrease throughout the entire time of the experiment, as did the inorganic phosphorus. Hence, the differences found for calcium and cholesterol, on the one hand, were largely quantitative, while those for inorganic and lipoid phosphorus (lecithin), on the other, were both quantitative and qualitative. Moreover, while, among animals living out-of-doors, there was evidence of a high order of correlation, among those living indoors, there was little or no correlation between the changes that occurred in the amounts of the several substances studied.

The highest degree of correlation was found to exist between calcium and inorganic phosphorus, Table III, Section (*c*), giving a value of -0.864 ± 0.047 for mean values of individual animals.

Finally, the results obtained warrant the conclusion that the difference in environment of the 2 groups of animals was sufficient to produce a definite effect on the chemical composition of the blood and on the mechanism coordinating the variations of the substances studied.

SUMMARY.

A series of determinations of inorganic phosphorus, calcium, cholesterol, and lecithin were made on a group of 10 animals living in the laboratory from Oct. 27, 1927, to May 17, 1928. A marked difference in both the trend and absolute values was noted in animals living in the laboratory when compared with the values obtained for animals living out-of-doors. With animals living in the open, the trend of variation for calcium was found to be the same. However, animals living in the laboratory maintained a higher level over the same period of time. The inorganic phosphorus and lecithin both showed a marked decrease and both maintained a lower level than was found in animals just received from the dealer. The cholesterol content of whole blood exhibited a similar trend in both groups of animals. However, it was found that animals living in the laboratory maintained a lower level than animals living out-of-doors.

The mean value for calcium was found to be 15.7 ± 0.05, for inorganic phosphorus 4.65 ± 0.05 mg. per 100 cc. of blood serum, and for cholesterol 58.2 ± 0.39 and lecithin 118.4 ± 1.13 mg. per 100 cc. of whole blood.

The coefficients of correlation having the highest degree of mathematical significance were obtained from the mean values for individual animals.

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THE RELATION OF FREQUENCY TO THE PHYSIOLOGICAL EFFECTS OF ULTRA-HIGH FREQUENCY CURRENTS.

By RONALD V. CHRISTIE, M.B., Ch.B., and ALFRED L. LOOMIS.

(From the Loomis Laboratory, Tuxedo, and the Hospital of The Rockefeller Institute for Medical Research, New York.)

(Received for publication, October 15, 1928.)

It is known that the physiological effects of alternating currents can be profoundly changed by changes in frequency. D'Arsonval (1) first showed that as the frequency of alternations is increased a point is reached where stimulation no longer occurs, and the subject merely feels a sensation of warmth and prickling in the skin. This critical frequency lies between 5,000 and 10,000 alternations per second, depending on the strength of the current (2). As the frequency is still further increased, the prickling sensation disappears till at 10^6 cycles per second heat production is the only effect which can be demonstrated. Accompanying the change in the physiological response with an increase of frequency, there is a change in the path of the current through the tissues. Whereas low frequency alternating currents confine themselves almost solely to the extracellular fluids, at a frequency of 10^6 the current actually penetrates the living cell. It does this by reason of the thinness of the dielectric layer surrounding the cell, which transmits the current by means of its capacitance (4). The heat produced at these frequencies can be wholly accounted for on the basis of ohmic resistance and dielectric loss (3).

The modern development of the vacuum tube oscillator has opened up a new field, as it has made it possible to investigate frequencies ranging from 1,000,000 to 158,000,000 and more cycles per second (300 meters to 1.9 meters). Very little analytical work has been done on the biological effects of these currents but it is believed that they differ fundamentally from those produced by any other type of electric current. The literature on the subject is apparently confined to three papers:

Gosset and his coworkers (5) in 1924 published a report on the effect on plant tumours of radiations from a vacuum tube oscillating at 150,000,000 cycles per

second. They observed that massive exposures to these rays killed various types of plant tumours, death being preceded by an acceleration in their rate of growth. More recently Schereschewsky (6) has studied the effect of these rays on mice. With the apparatus at his disposal he was able to subject mice to lethal exposures at frequencies varying from 8.3 to 135 million cycles per second. He claimed to have shown that the lethality was maximal when the frequency lay between 20 and 80 million cycles. This phenomenon he ascribed to some selective action of these wave-lengths, suggesting that this might be in the form of electro-mechanical vibration of the living cell caused by the rapid alternations in polarity of the field. He also observed that while the rectal temperature of a live mouse could be raised 5–6°C. by these currents, that of a freshly killed mouse could only be raised 0.1–0.7°C. in a similar length of time. This he believed was evidence to show that the heating effect with these currents was different from the diathermic effect observed at lower frequencies.

Having shown that there might be a differential action upon tissue cells with regard to frequency, Schereschewsky proceeded to investigate the effects of these radiations on transplantable tumours. A total of 403 mice with the mouse sarcoma were treated by radiation at a frequency of about 67,000,000 cycles per second,—the frequency which had been found to be most lethal to healthy mice. The radiations were strictly localized to the mass of the tumour, so that any systemic effect was excluded. Out of the total of 403 mice treated only 5.5 per cent actually died of the tumour, 23 per cent recovered, and the remainder died from other causes. In 203 control mice, no case of spontaneous recession of the tumour was observed. Somewhat similar results were obtained with chickens that had Rous sarcoma. Microscopic sections of tumours removed after radiation gave a picture resembling coagulation necrosis.

In a small series of experiments a frequency of 135,000,000 cycles was employed, but these radiations were found to have no demonstrable effect on the tumours, which seems to him to support his hypothesis that a specific frequency may be essential.

In short, Schereschewsky thinks that there are certain wave-lengths which have a specific lethal action on living cells. This specific band of frequency lies, he says, between 20,000,000 and 80,000,000 cycles per second (*i.e.* between wave-lengths of 15 and 3.8 meters). This same band he says also destroys transplantable tumours in mice and frequencies outside this band have no demonstrable effect.

These observations, if confirmed, may obviously have a wide range of interest in biology and medicine. Previous work, however, had convinced us that the biological effects of currents of lower frequency could be explained solely on the basis of heat production. It was of great interest to us, therefore, to find out whether the same was true

of currents of higher frequency, or whether some specific action on living cells existed.

Apparatus.

1. Generation of High Frequency Oscillations.

As is well known, the natural frequency of an oscillating circuit is made as high as possible by reducing the value of its inductance and capacity to the lowest possible limit. The technique of constructing oscillating circuits of ultra-high frequency (above 100,000,000 cycles per second) radiating relatively large amounts of power is rather new and has not been generally described outside of the literature dealing with radio transmission.

To obtain a frequency of 150,000,000 cycles per second (2 meters wave-length) we use two No. 852 radiatrons. These tubes are rated at 75 watts each and are especially designed for high frequency work as the grid capacity has been made as small as possible and this is the only capacity in our oscillating circuit. The inductance is a short straight piece of heavy copper tubing 6 inches long directly connecting the grids of the two tubes (see Fig. 1). To lessen the frequency it is only necessary to substitute a longer piece of copper tubing.

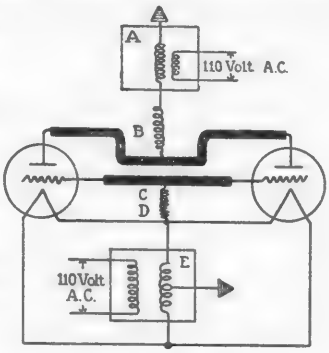

FIG. 1. Circuit used for wave-lengths less than 14 meters: *A*, plate transformer delivering 2,000 volts to the plates. *B* and *C*, radio frequency chokes. *D*, 10,000 ohm grid leak. *E*, filament transformer.

The plates are connected by another piece of copper tubing as shown in the diagram and the inductive coupling to the grid is varied by rotating the U-shaped piece until the position of maximum power output is obtained. These tubes radiate a very considerable amount of power. An ordinary 50 watt tungsten lamp with a small copper loop fastened to the base is lighted to full brilliancy when held near the oscillator. If a receiver is made by taking a thermoammeter and fastening two short pieces of copper tubing to the binding posts thus making an antenna 1 meter long (*i.e.* a half wave-length) with the meter in the center and placed 30 feet from the oscillator a deflection of over 50 milliamperes is observed on the meter.

The exact wave-length of the radiation is measured by the usual method, on 'Lecher wires." Two parallel wires are strung near the oscillator and the system

of standing waves induced in these wires is determined with a neon light and a movable bar connecting the wires; the distance between each nodal point is a half wave-length. For wave-lengths longer than 14 meters we have used the conventional Hartley circuit with a 250 watt tube.

2. Utilization of Oscillator Output.

To utilize the oscillator output a simple resonance circuit was used in which the size of the condenser plates was kept constant, and the inductance varied to attain resonance.

At wave-lengths of 6.0 to 1.9 meters an ammeter was placed in the center of the inductance and at 14 to 38 meters the ammeter was placed adjacent to one plate of the condenser. (An exception was made in this procedure in the first series of experiments when at 2 meters the ammeter was placed adjacent to the condenser plate.) By inserting any poor dielectric, such as a mouse, in the electrostatic field between the plates of the condenser there will be considerable dielectric loss, leading to the production of heat. In other words, a mouse suspended in this field will be subjected to an alternating current of the same frequency as that emitted by the vacuum tube oscillator. As the electrostatic field between the condenser plates is certainly not a homogeneous one, it was obviously necessary to maintain the position of the mice in the field constant with regard to the condenser plates. To do this they were inserted into a Pyrex glass tube of such dimensions that a 20 gm. mouse was able to crawl into the tube but could not move from side to side. With the mouse in the fundus of the tube, its movements were still further limited by the insertion of a glass disc supported by a rod which was embedded in a rubber stopper in the open end of the tube (Fig. 2). It was found that the condensation of moisture from the mouse on the walls of the tube could lead to considerable heating of the glass, when the current was turned on. The condensation of moisture was especially marked round the animal's buttocks. To prevent this the air used for ventilation was warmed and kept as dry as possible (*vide infra*) and a small vent for the escape of air was made through the cork as well as at the fundus of the tube (Fig. 2). By this means more ventilation was brought to bear on the animal's buttocks than elsewhere and heating of the glass was satisfactorily prevented. The tube containing the mouse was inserted between the plates so that it lay horizontally, with the mouse as nearly as possible in the center of the electrostatic field.

3. Measurement of Current.

In a resonance circuit such as has been described we do not believe that, with changing frequency, an ammeter gives any indication of the intensity of the radiations between the condenser plates. The inductive part of the circuit has considerable capacitance, so that the

actual amount of current flowing in different parts of the inductance will vary widely. The ammeter will give a maximum reading at a point in the center of the inductance and a minimum reading at the point where the inductance joins the capacitance. Even if the ammeter is kept in the center of the inductance, a change in frequency would of necessity alter the amount of capacitance in the inductive part of the circuit. This in turn would alter the ratio of the current flowing in the ammeter to the intensity of the field between the condenser plates. From theoretical principles it is then obvious that any change in the inductance or capacitance of a circuit renders an ammeter reading meaningless with regard to the amount of energy delivered between the plates of the condenser. As will be shown later, it is easy to vary the ammeter reading and the energy between the plates quite independently by changing the inductance, capacitance, or position of the ammeter. It is only when the position of the ammeter and the structure of the circuit are kept constant that the ammeter reading is of any significance and this is obviously impossible if the frequency is to be changed. Also, any "skin effect" in the thermocouple will introduce an error, the magnitude of which will change with frequency. It was evident therefore that some method must be devised whereby the energy between the condenser plates could be measured directly. To do this we used as our standard the heat produced in a simple electrolyte when suspended between the condenser plates.

The rate of heat production in an electrolyte under these conditions will constitute a direct measurement of the radiant energy between the plates, irrespective of frequency (7).

In our first series of experiments on mice, we used as our standard the rate of heat production in 100 cc. of $\frac{M}{10}$ NaCl in a glass cell. By this method successive observations under as far as possible similar conditions gave checks which only corresponded to within ± 10 per cent. This was due in part at least to irregularities in heat production at the phase boundary between the electrolyte and air, and to the fact that the fluid had to be stirred. To obviate these sources of error a device like a large thermometer was constructed with the bulb containing 20 cc. of $\frac{M}{20}$ NaCl, coloured with safranine, and the stem graduated to from 15–35°C. When the bulb of the thermometer was suspended in the field between

the condenser plates, it was found that at any frequency checks agreeing within ±1 per cent could be obtained provided the position of the thermometer and the various constants of the circuit remained unchanged. In the first series of observations $\frac{M}{10}$ NaCl in a cell was used to gauge the strength of the radiations to which the mice were being submitted. At each frequency the tuned circuit was so arranged that with the cell in position, the $\frac{M}{10}$ NaCl heated at a rate of approximately 0.5°C. per minute. The cell was then removed and the mice subjected to the radiations. In the second series of observations a somewhat different

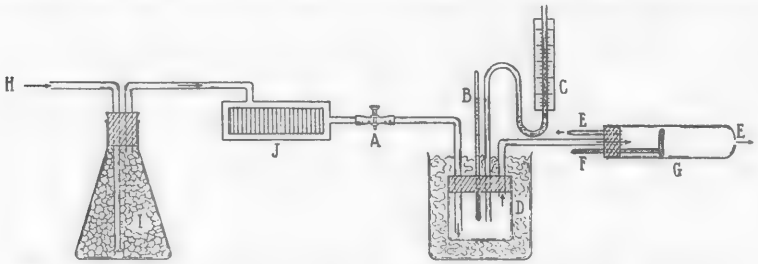

FIG. 2. Apparatus used to maintain a mouse under constant atmospheric conditions while suspended in the magnetic field. *A*, screw clamp to regulate air pressure. *B*, thermometer. *C*, water manometer. *D*, insulated mixing chamber. *E*, openings for escape of air. *F*, rod to keep mouse in position. *G*, mouse holder. *H*, source for air pressure. *I*, calcium chloride. *J*, electric heater.

procedure was adopted. During each experiment the $\frac{M}{20}$ NaCl thermometer was suspended vertically in the field, immediately above the center of the mouse so that both were being heated simultaneously. The heat produced in the thermometer was taken as an index of the strength of the field between the condenser plates.

4. Maintenance of Constant Effective Temperature.

Since it is known that the production of heat is one of the effects produced by these radiations, it was thought advisable to keep the atmospheric conditions around the mouse as far as possible constant.

For this purpose air of constant humidity and temperature was forced through the mouse chamber at constant pressure (Fig. 2). The humidity was reduced to approximately zero by passing the air through calcium chloride. The tem-

perature was then raised by means of an electric heater to from 22–24°C. This warm dry air was then passed into the mouse chamber at a pressure of 30 mm. of water. The air was especially suitable for ventilation as it was below the "comfort zone" (8), and prevented moisture from condensing on the walls of the mouse chamber.

Even under these standardized conditions it was found that a considerable variation existed in the response of mice to any given quantity of current. Some mice made efforts to escape from the chamber and soon died while others compensated by quietly panting and survived for a longer time. Since variations due to this individual factor diminished as the current strength was increased, we decided to use more powerful radiations than did Schereschewsky. Also since the effectiveness of the heat-regulating mechanism of a mouse varied greatly with slight changes in the current strength, we believe that measurements of the amount of current required to kill the animal in a given length of time are of more significance than measurements of the time taken by a fixed quantity of the current to kill the animal. However, both methods were used in the experiments about to be described.

5. Measurement of Temperature in Mice.

To obtain a record of temperature changes in a mouse, special thermometers were constructed, of such dimensions that they could be inserted into the mouse's rectum. This was perfectly satisfactory for a live mouse, but it was early found that with a dead mouse which had been subjected to the radiations, a wide range of error existed. The distribution of heat was very irregular, so that if the bulb of the thermometer were held just inside the rectum a higher temperature was recorded than if it were inserted into the abdominal cavity. In the first series of experiments the rectal thermometer was used to measure the temperature after death. Care was taken to have the bulb lying in the abdominal cavity, not touching the spine. Even with these precautions a considerable margin of error undoubtedly persisted in the dead mice which were radiated, as a slight movement of the thermometer often caused a change of as much as 2°C.

To obviate these sources of error a method was devised whereby the temperature of the mouse could be estimated calorimetrically.

For the purpose a Dewar flask was used, of such dimensions that when closed with a rubber stopper, it could just hold a 20 gm. mouse and 130 cc. of water. In the stopper were two openings through one of which was inserted a thermometer and through the other a glass tube to allow for the escape of air when

the stopper was inserted. The technique for estimating the caloric increase in a mouse after subjecting it to the radiations was as follows: The rectal temperature of the mouse was taken before radiation, and immediately after death the animal was plunged into the Dewar flask which had been filled with 120 cc. of water at room temperature. The stopper was then inserted and the flask allowed to stand for 20 minutes, being gently agitated every 5 minutes. By this time an equilibrium had been reached and the increase of temperature in the water could be read. The weight and specific heat of the mouse being known, it was possible to calculate the increase in calories brought about by exposure to the radiations. This was done by subtracting the number of calories as calculated from the rectal thermometer reading, from the number of calories as measured in the calorimeter. From the caloric increase, the temperature increase could be calculated. As no figures were available for the specific heat of mice, this had to be determined directly. To do this 8 dead mice of known weight were placed in an incubator at 40°C. for 6 hours. They were then placed in the calorimeter and the increase of temperature in the water observed, as described above. It was found that on an average the observed temperature increase was 69.2 per cent of what one would expect if an equal weight of water at 40°C. were poured into the flask. The actual figures obtained varied from 64.7 per cent to 73 per cent, but as all the data obtained by this method were used in a relative rather than in an absolute manner, a mean of 70 per cent was considered sufficiently accurate for our purpose. When we say that 0.7 represents the specific heat of mice we realise that this factor includes corrections for various sources of error in the method, but we consider this to be of advantage, since it is in the increase of temperature and not in the specific heat of mice that we are interested.

EXPERIMENTAL.

The experiments about to be described naturally fall into three groups which can conveniently be considered separately. The first series of experiments were planned to find if the lethal nature of these radiations was strictly proportionate to their intensity, irrespective of frequency, or if certain frequencies had a specific lethal action on living cells. These experiments indicated the need for certain refinements of technique which were incorporated in the second series. The third series was planned to find the actual cause of death in animals subjected to these radiations.

First Series.

The procedure in this series of experiments consisted of subjecting live mice to the radiations by means of the tuned auxiliary circuit and ventilated mouse chamber described above. The amount of current flowing in the auxiliary circuit

was measured by means of an ammeter and also by the amount of heat produced in 100 cc. of $\frac{M}{10}$ NaCl (*vide ante*). The condenser plates were kept about 3 cm. apart. Temperature measurements were made on the mice before and after radiation by means of a rectal thermometer. In all, four wave-lengths were investigated: 2, 6, 14, and 38 meters. In the last three of these several different current strengths were used. At each current strength, from 2 to 10 live mice and from 3 to 13 dead mice were radiated and the temperature increase in each

TABLE I.

Effect on Live Mice of Lethal Exposures, and on Dead Mice of Exposures of Similar Duration and Intensity. First Series.

Frequency $\times 10^6$	Wave-length	Current strength	Rate of heating of M/10 NaCl in cell per min.	Number of mice		Average duration of exposure		Average temperature increase		Average temperature increase per min.	
				Alive	Dead	Alive	Dead	Alive	Dead	Alive	Dead
cycles per sec.	*meters*	*amperes*	°C.			*min.*	*min.*	°C.	°C.	°C.	°C.
150	2	1.35	0.54	9	12	4.79	4.71	4.99	5.77	1.05	1.39
50	6	1.6	0.08	2	2	20.8	20.5	4.3	8.1	0.21	0.40
50	6	3.0	0.47	10	13	4.90	4.86	5.17	5.17	1.05	1.06
21.4	14	1.0	0.40	6	6	5.05	5.11	4.28	3.6	0.86	0.70
21.4	14	1.6	0.98	8	7	3.76	3.71	4.63	3.97	1.21	1.08
21.4	14	1.8		4	5	3.47	3.36	5.62	4.08	1.55	1.20
21.4	14	2.2		5	7	2.62	2.58	5.18	3.95	2.01	1.76
7.9	38	0.55	0.45	6	3	5.3	5.3	5.35	5.30	1.01	1.00
7.9	38	0.65		2	—	4.25	—	5.35	—	1.25	—

mouse was recorded. It was found at all frequencies that, within the limits of error, the rate of heating of the $\frac{M}{10}$ NaCl was proportional to the rate of heating of live and dead mice, and also to the time taken to kill the live mice (Table I). The amperage on the other hand bore no relationship to any of these figures when the frequency was changed. For example, with the current of such a strength that the salt solution heated at 0.4–0.54°C. per minute, mice were killed in from 4 to 5 minutes at all frequencies and their rate of heating was approximately 1°C. per minute. The amperage, however, varied from 0.55 to 3.0 as the frequency was changed.

The rate of heat production in dead mice was found to be on an average approximately the same as that of live mice at all frequencies. This is contrary to what was found by Schereschewsky. The dis-

crepancy can readily be explained by the fact that we deliberately allowed the dead mice to cool down to room temperature before being radiated, while Schereschewsky radiated them immediately after death. We found that a dead mouse at 38°C. loses heat at a rate of more than 1°C. per minute, so that in Schereschewsky's experiments heat was being lost by the mice almost as rapidly as it was being generated in them. This obviously only applied to dead mice, as the metabolism of a live mouse is in itself sufficient to maintain a constant temperature. To show experimentally that this explanation is valid, we subjected three groups of mice to radiations at 2 meters wave-length, the auxiliary circuit and the average duration of exposure in each group being kept constant. The first group consisted of live mice, the second of freshly killed mice, and the third of dead mice which had been allowed to cool to room temperature. The temperature increase in these three groups averaged 4.42°C. for the live mice, 1.2°C. for the freshly killed mice, and 5.9°C. for the cooled dead mice.

It is apparent from this experiment that Schereschewsky's observation that currents of certain frequencies will raise the temperature in living mice to a greater degree than in dead mice can be explained not on the basis of specific properties of the radiations, but simply by the fact that the dead mice lost heat during exposure to the rays while the live mice did not.

Second Series.

The technique used in this series was similar to that used in the first with three exceptions.

The temperature of the mice after radiation was measured calorimetrically; the strength of the current was measured with the $\frac{M}{20}$ NaCl thermometer; and measurements were made of the distance between the plates of the condenser in the auxiliary circuit. As we believe that the observations in this series have a much higher degree of accuracy than those in the first series, they will be given in greater detail. Observations were made at 5 wave-lengths, 1.9, 3.2, 6.0, 14.0, and 36.1 meters. At each frequency a group of mice were radiated with the current at such a strength that the $\frac{M}{20}$ NaCl thermometer was heated at a rate of 0.5°C. per minute. Under these circumstances it was found that between 6.0

and 36.1 meters the animals were killed in from 3 to 5½ minutes while at 3.2 meters they were killed in from 6 to 12 minutes and at 1.9 meters in from 8 to 13½ minutes (Table II, Fig. 3). The rate of heat production in these mice and also in dead mice exposed to the same amount of current was found to bear the same relationship to the heating of $\frac{M}{20}$ NaCl at the various frequencies. At 36.1, 14.0, and 6.0 meters the rate of heating in the groups of live mice averaged 1.95°, 1.88°, and 1.90°C. per minute and in the groups of dead mice 2.64°, 2.75°, and 2.55°C. per

TABLE II.

Effect on Live Mice of Lethal Exposure and on Dead Mice of Exposures of Similar Duration and Intensity. Second Series.

Frequency × 10^6	Wave-length	Current strength	Rate of heating of M/20 NaCl per min.	Distance between plates	Number of mice		Average duration of exposure		Average temperature increase		Average temperature increase per min.	
					Alive	Dead	Alive	Dead	Alive	Dead	Alive	Dead
cycles per sec.	*meters*	*amperes*	°C.	*cm.*			*min.*	*min.*	°C.	°C.	°C.	°C.
158	1.9	4.3	0.52	4.2	6	6	9.6	10.1	5.59	14.5	0.563	1.46
158	1.9	3.4	0.59	2.9	2	—	7.2	—	6.60	—	0.895	—
158	1.9	3.8	0.65	2.9	6	—	4.83	—	5.53	—	1.115	—
158	1.9	4.2	0.74	2.9	2	—	3.35	—	6.40	—	1.90	—
93.7	3.2	1.4	0.37	4.9	2	—	29.8	—	4.90	—	0.16	—
93.7	3.2	1.7	0.53	4.9	7	6	9.8	10.18	5.96	11.9	0.626	1.16
93.7	3.2	2.0	0.65	4.9	2	—	6.6	—	4.70	—	0.73	—
93.7	3.2	4.9	0.74	4.9	9	—	5.1	—	4.60	—	0.94	—
50.0	6.0	1.5	0.48	2.9	6	6	4.58	4.53	8.53	11.50	1.90	2.55
21.4	14.0	1.2	0.51	2.9	6	6	4.1	3.97	7.69	10.87	1.88	2.75
8.3	36.1	1.0	0.30	4.2	3	3	11.2	11.6	8.60	11.43	0.79	1.00
8.3	36.1	1.0	0.49	2.9	6	6	4.35	4.73	8.27	11.83	1.95	2.64
8.3	36.1	1.05	0.60	2.9	2	—	2.25	—	4.70	—	2.58	—

minute. At 3.2 and 1.9 meters, however, the live mice heated at 0.63° and 0.56°C. per minute and the dead at 1.16° and 1.46°C. per minute respectively (Table II, Figs. 4 and 5).

The significance of these figures will be discussed later, but, in brief, it would appear that the lethal nature of the radiations and the amount of heat that they generate in mice, remain constant up to a frequency of about 50,000,000 cycles (6 meters wave-length). When this frequency is exceeded, however, both the heat production and the lethality diminish. Observations of a similar nature to those de-

scribed above but with other rates of heating of the saline thermometer pointed to the same conclusions (Table II). For additional confirma-

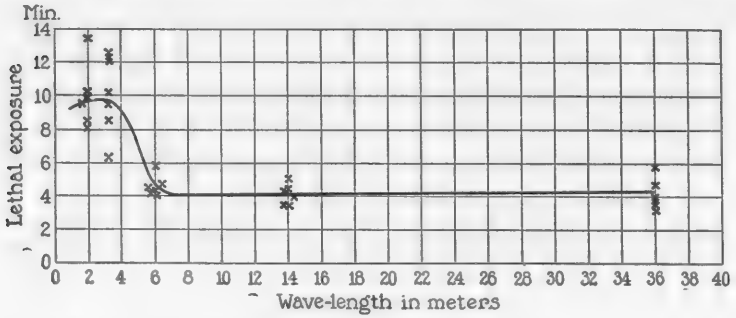

FIG. 3. Curve showing duration of lethal exposure for mice. The ordinates represent time in minutes; the abscissæ the wave-length in meters of the radiations to which the mice were exposed. At each wave-length the radiations were of such strength that the $\frac{M}{20}$ NaCl thermometer was heated at the rate of 0.5°C. per minute.

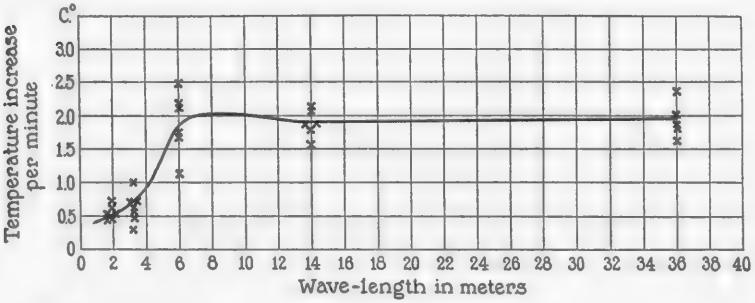

FIG. 4. Curve showing the rate of heating in mice when subjected to a lethal exposure. The ordinates represent the rate of heating in °C. per minute; the abscissæ the wave-length in meters of the radiations to which the mice were exposed. At each wave-length the radiations were of such strength that the $\frac{M}{20}$ NaCl thermometer was heated at the rate of 0.5°C. per minute.

tion observations were made on the amount of current required to kill the mice in approximately 5 minutes. Under these circumstances the

saline thermometer heated at a rate of 0.5°C. per minute at 36.1, 14.0, and 6.0 meters, at 0.74°C. per minute at 3.2 meters, and at 0.65°C. per minute at 1.9 meters (Table II, Fig. 6). This diminution in lethality at the higher frequencies was also suggested by the experiments made in the first series, although the change was within the limits of experimental error.

In this series of experiments we also found that the elevation of temperature in dead mice was considerably greater than that found in

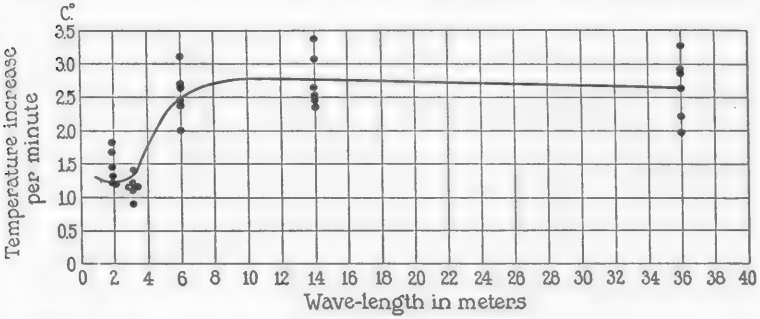

FIG. 5. Curve showing the rate of heating in dead mice when subjected to the same exposures as had been shown to be lethal to live mice. The ordinates represent the rate of heating in °C. per minute; the abscissæ the wave-length in meters of the radiations to which the mice were exposed. At each wave-length the radiations were of such strength that the $\frac{M}{20}$ NaCl thermometer was heated at the rate of 0.5°C. per minute.

live mice. The average increase in 59 live mice was 6.31°C. while the average increase in 33 dead mice subjected to an equal amount of the current was 12.01°C. The discrepancy is what one would expect if heat were being generated in both the live and dead mice at the same rate. Heat loss in the dead mice was reduced to a minimum, while in the live mice the heat-regulating mechanism was allowed to operate unhampered. It will be remembered that in the first series we found live and dead mice to heat at much the same rate. The method used for making temperature measurements was admittedly inaccurate in this series, especially when dead mice were employed.

As in the first series so in this, we could find no direct relationship between the amperage, the lethal effect on mice, and the heating of saline. It will be seen from Table II that the amperage as shown by the meter may be varied by altering the constants of the tuned circuit while the current passing between the plates as measured by the rate of heating of the saline thermometer remains unchanged or changes independently. This is in accord with the theoretical considerations given earlier in the paper, and demonstrates the fallacies underlying the use of the ammeter in an investigation of this type.

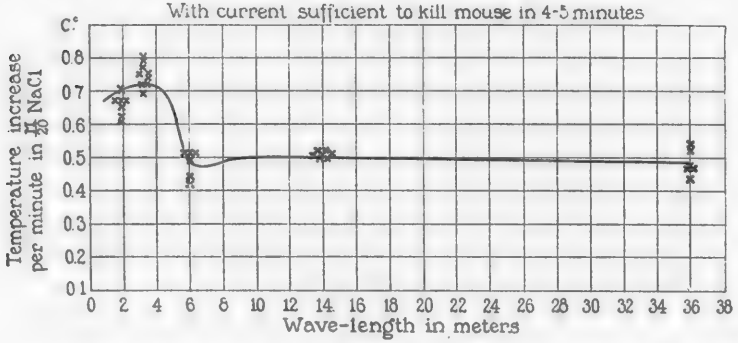

FIG. 6. Curve showing the rate of heating in the $\frac{M}{20}$ NaCl thermometer, when subjected to radiations of such strength that mice were killed in from 4 to 5 minutes. The ordinates represent the rate of heating in °C. per minute; the abscissæ the wave-length in meters of the radiations to which the mice and thermometer were exposed.

Third Series.

It has been shown that radiation with these currents is always accompanied by heat production. In the 120 mice which are included in Series I and II the increase in temperature observed when mice were killed by the radiations ranged from 2–9°C. with an average of 5.7°C. (Fig. 7). Before we could ascribe the death of these animals to the increase of temperature it was obviously necessary to find exactly what increase could be tolerated by the normal mouse. To do this two series of experiments were performed.

In the first experiment 4 mice were placed in an incubator at 60°C. and immediately after death, which occurred in 8 to 10 minutes, the temperature increase was found to average 6.65°C. In another series 6 mice contained inside

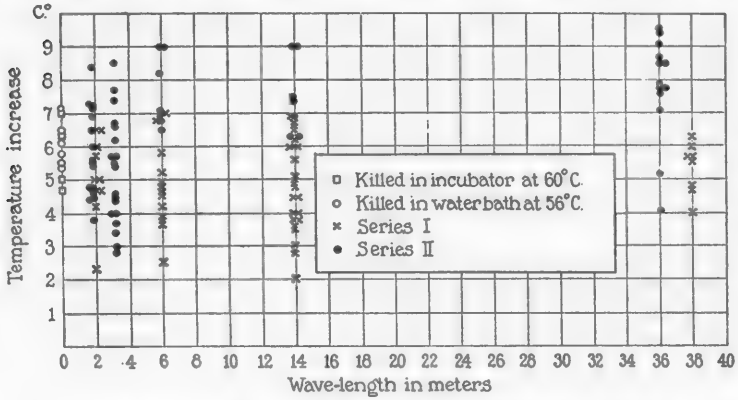

FIG. 7. The black dots and crosses represent the temperature increase at death in mice which had been subjected to lethal exposures of the radiations. The circles and squares represent the temperature increase at death in mice killed by exposure to environmental heat. The ordinates represent the temperature increase in °C.; the abscissæ the wave-length in meters of the radiations to which the mice were exposed.

TABLE III.

Effect of Sublethal Exposures on Frogs.

Wave-length	Current strength	Duration of exposures	Temperature increment					Highest temperature reached
			1st exposure	2nd exposure	3rd exposure	4th exposure	5th exposure	
meters	*amperes*	*min.*	°C.	°C.	°C.	°C.	°C.	°C.
2	1.35	10	10.04	10.03	8.10	7.60	—	34.2
6	3.0	5	15.3 (10 min.)	8.05	6.2	7.3	9.4	37.0

ventilated tubes were introduced into a water bath at 56°C. The average time taken was 4.47 minutes and the temperature increase 6.08°C. These experiments are represented in Fig. 7. The close agreement between the temperatures leading to death in the experiments and resulting from the radiations is apparent.

Several experiments were performed to show that these currents are harmless apart from their heating effect. In the first experiment 2 mice were subjected to 2 meter radiations of a strength to produce death in about 5 minutes. At 2 minute intervals the current was stopped for 2 minutes. Each mouse readily endured a total of 10 minutes radiation. Because of the poor circulation in the tail, overheating of this organ occurred with the result that it was lost (9).

In the next experiment frogs were used.

The technique of exposure to the currents was the same as that used with mice. Radiation was continued for from 5 to 10 minutes, when the groin temperature was taken, and the frog immersed in cold water. After the animal had cooled, the procedure was repeated. At 2 meters 4 exposures were without harmful effect although the temperature rose from 7.5–10°C. at each exposure. At 6 meters 5 exposures were borne although at the first, a temperature of 37°C. was reached and the animal lay as if dead (Table III). Thus if the systemic temperature be kept within tolerable limits, no ill effects result from even prolonged exposures to these radiations.

DISCUSSION.

The results of these experiments are definitely at variance with those obtained by Schereschewsky. They show that the lethal nature of these radiations is proportionate to the intensity of the field up to a frequency of about 50,000,000 cycles. At frequencies higher than this, the lethality of the radiations appears to diminish. It must be realised that the standard of current strength was the intensity of the electromagnetic field and not necessarily the amount of current induced in the mouse. We believe that in this lies the explanation of the apparent diminution in lethality and that changes in the dielectric constant of the mouse cause a diminution in the amount of current induced in it. From Debye's equation for the dielectric constant as a function of frequency and viscosity it is apparent that in the region of the shorter wave-lengths it is possible that the highly viscous elements of the mouse (fat, epidermis, bone, etc.) begin to play an important part in its dielectric properties, and either diminish or increase the power loss. An analysis of the causes of deviation in this region is impossible, both because the dielectric properties of the constituent parts of the living organism are unknown (10) and the power loss in simple solid dielectrics has not yet received adequate theoretical

treatment. It should be noted, however, that at wave-lengths less than 6 meters a mouse ceases to behave in the same manner as an electrolyte, since a deviation of the type found requires the introduction of heterogeneity. Changes in the dielectric constant of the mouse at these very high frequencies would entail a readjustment of the field in the condenser, causing a change in the ratio of power loss between the mouse and the saline thermometer. Curves of the type observed in Figs. 3 to 6 might easily be obtained with a system more simple than a mouse, and hence although it is not at present practicable to analyse the factors causing changes in the dielectric properties, the phenomenon in essence is not at all incomprehensible in its physical aspect. The results of our experiments certainly do not suggest that any specificity exists with regard to the action of any particular wave-lengths on living cells. They do indicate that the lethal effect of these radiations is diminished at the very low wave-lengths, but we believe that this is due to less current being induced in the mouse.

We have mentioned the errors involved in the use of a thermocouple ammeter for measurement of the intensity of the eletrostatic field between the condenser plates. In the auxiliary circuit used by Schereschewsky the error due to skin effect would increase with the frequency while the error due to the capacity of the inductance would decrease with frequency. In his experiments he kept the milliammeter reading constant but the intensity of the field between the plates was certainly changing. Since this change would be, in part at least, governed by the two factors mentioned above, the type of curve which he obtained for the lethal effect of these radiations on mice, might only represent changes in the intensity of the field.

The lethal effect of these currents can be fully accounted for on the basis of heat production. We believe that the production of this heat is strictly analogous to that observed in diathermy, being generated by the resistance of the tissues and dielectric loss. Considerable evidence is at hand to support this statement. Lethal doses of the current, at all frequencies are accompanied by the same degree of temperature elevation. Heat production in dead mice parallels that in live mice irrespective of frequency. Sublethal doses given to mice are without ill effects apart from those which can be accounted for by

the local accumulation of heat. Animals which are poikilothermic can be given prolonged exposures without any harmful effect provided the systemic temperature be kept below the lethal point. Moreover, we believe that the data presented by Schereschewsky can also be explained on the basis of heat production by the induced alternating currents. Our interpretation of what he believes to be a specific band of wave-lengths has already been described and a simple explanation has been furnished for the fact that in his experiments dead mice heated less readily than live ones. The effect of these rays on tumours may also in all probability be accounted for on the basis of heat production. Schereschewsky states that the tumours did not feel hot but on the other hand the microscopic picture of the tumours after radiation suggested coagulation necrosis. It should be borne in mind that any heat developed in the tumour would rapidly be disseminated after the current was turned off. Direct temperature measurements in the substance of the tumour during radiation would be necessary to rule out the probability that the structural changes observed were not the result of heat. In fact in our opinion the burden of proof still lies on those who claim any biological action of high frequency currents other than heat production.

SUMMARY AND CONCLUSIONS.

1. Biological effects of electromagnetic waves emitted by a vacuum tube oscillator have been studied at frequencis ranging from 8,300,000 to 158,000,000 cycles per second (1.9 to 38 meters wave-length).

2. The effects produced on animals can be fully explained on the basis of the heat generated by high frequency currents which are induced in them.

3. No evidence was obtained to support the theory that certain wave-lengths have a specific action on living cells.

4. At frequencies below 50,000,000 cycles, the effect of these radiations on animals is proportionate to the intensity of the electromagnetic field. As the frequency is increased beyond this point, the amount of induced current is diminished and the apparent lethality of the radiation is decreased. This can be explained by changes occurring in the dielectric properties of tissues at low wave-lengths.

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MATERIAL FROM LYMPH NODES.

IV. The Heterology of Lymphoid Tissue with Special Reference to the Monocyte—Supravital Studies.

By CLAUDE E. FORKNER, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

Plates 21 to 24.

(Received for publication, October 8, 1928.)

The object of this study was to make a complete survey of lymph nodes by the supravital technique. The method applied to the bone marrow, connective tissues, and blood has yielded information of a new sort. There is no record in the literature of its application to all of the lymph nodes of the body and a correlation of the findings with the histology of fixed tissues.

Cunningham, Sabin, and Doan (1) studied the mesenteric lymph nodes of rabbits by means of punctures of these tissues and the withdrawal of cells. In their studies of living cells so obtained, they found over 90 per cent of the cells to be of the lymphocytic series. The other 10 per cent were made up of a few clasmatocytes, a few polymorphonuclear leucocytes, and an occasional myelocyte. In no experiment in which blood vessels were not ruptured by the punctures did they find any typical monocytes.

McJunkin (2) was the first to study the peripheral lymph nodes by means of the supravital technique. He reported studies on inguinal, iliac, and axillary lymph nodes of rabbits. In these tissues he found great numbers of "rosette cells." These cells, he believed, arose from the "reticular lymph vessel endothelium." From observations of these three groups of lymph nodes, he drew the conclusion that lymph nodes in general contain monocytes in abundance.

Jordan (3) studied fixed sections of lymph nodes of four rabbits. He states that a description of the cervical group may suffice as applying also to modified nodes of the abdominal group. He noted no differences in the types of cells found in the mesenteric and superficial nodes. He believes that the lymph nodes are a source of proliferation of red blood cells by a process of conversion of lymphocytes into erythrocytes.

Maximow (4) stated that two varieties of cells must be distinguished among the reticular stroma of lymphoid tissue. The first are the reticular cells—large stel-

late or spindle-shaped elements, with more or less abundant protoplasm and oval, pale nuclei containing little chromatin. These, he believes, are the fixed histiocytes and belong to that group of cells scattered all over the body and represented in the common connective tissues by the macrophages or clasmatocytes. The other cells form a reticular syncytium, sheathing the reticulin fibers. Their nuclei are pale and inconspicuous, and they have a small amount of protoplasm. They do not phagocytize and do not store vital dyes. These elements keep their mesenchymal prospective potencies and may form lymphocytes or histiocytes. In the meshes of the reticulum free macrophages and lymphocytes were found.

Weidenreich, alone (5) and with Downey (6), made detailed studies of the germ centers. They found that even under physiological conditions there was extraordinary variation in the number of cells, the degree of basophilia, and relative volumes of nuclei and cytoplasm. These authors drew the conclusion that there is no special germ center cell, but that the cells of this region in the active phase consist essentially and almost exclusively of intermediate lymphocytes, among which may be scattered small lymphocytes and large lymphocytes. There are a few scattered, pale, oval nuclei between the lymphocytes which probably belong to the reticular syncytium. Along the capillaries of the germ centers, macrophages containing phagocytized material may be seen. In the resting phase the germ center is small and consists almost exclusively of small lymphocytes, with a few reticular nuclei of the reticular syncytium.

Materials and Methods.

In the work reported here, surveys of the lymph nodes have been made on 58 rabbits. Fourteen of these were normal animals taken directly from stock. The remaining material was derived from the routine autopsies of animals which had been subjected to various experiments in this laboratory. In these latter cases there was no apparent abnormality in the lymph nodes. Certain pathological states were studied, but the results of the observations are not reported in this communication. Most of these surveys have included studies of all of the lymph nodes of the body, whereas in some of the animals, selected nodes have been studied.

The groups of lymph nodes observed were as follows: popliteal, inguinal, iliac (flank), superficial axillary, deep axillary, superficial cervical, deep cervical, mediastinal, mesenteric (large mass), isolated mesenteric, and retroperitoneal. Peyer's patches, the thymus, and spleen were also studied. The nodes were dissected out from the living, anesthetized animal, or from rabbits immediately following death produced by the intravenous injection of from 10 to 20 cc. of air. It is important to obtain absolutely fresh tissue for supravital studies. Many of the cells die

within a few minutes of the death of the animal, or soon after removal from the body. Monocytes are usually the first cells to show evidence of degeneration and death, whereas clasmatocytes appear to survive for hours.

Scrapings were made from the cut surfaces of the nodes. There was usually sufficient fluid present in the lymph nodes themselves to suspend the cells obtained in the scraping. Supravital preparations were made by placing a small drop of the fluid from the scraping on a slide covered with a dried film of dye. A coverslip was immediately inverted, and the preparation was rimmed with vaseline and studied. The technique of making supravital preparations has been used by Rosin and Bibergeil (7, 8), Pappenheim (9), Cowdry (10), Simpson (11), Sabin (12), and others. The most extensive studies with this method have been carried out by Sabin and her pupils. The method has been described many times by the above authors and need not be repeated here in detail. The dyes used for these studies were neutral red alone or a combination of neutral red and Janus green. The neutral red stains cytoplasmic structures, such as specific granules, segregation granules or vacuoles, and phagocytized material. Janus green in dilute concentration is a specific stain for mitochondria.

Description of Cells.

Before giving a description of the difference in cytology of each of the specific groups of lymph nodes, there will be described all of the types of cells which were encountered in a complete survey of lymphoid tissue in the normal rabbit.

The element most commonly encountered in scrapings of all lymph nodes is the small lymphoid cell (Fig. 1). In addition there are intermediate and large lymphoid elements, monocytes, epithelioid cells, giant multinucleated cells with rosettes, histogenous basophils (mast cells), eosinophilic myelocytes, or perhaps more properly histogenous eosinophils, and clasmatocytes or macrophages. There also occur giant cells, with one or more nuclei and containing large, refractive, even sized, unstained granules, giant cells with one or more nuclei and containing uneven, refractive bodies together with peculiar sickle-shaped or fusiform structures, filling the entire cytoplasm, large cells containing in their cytoplasm bodies appearing like blood platelets, endothelial cells, primitive cells, and fibroblasts. Reference to Table I will give at a glance the essential characters and staining reactions of these cell types.

	Small lymphoid cell	Intermediate lymphoid cell	Large lymphoid cell	Monocyte ("rosette cell")	Epithelioid cell	Giant multinucleated cell with rosette	Histogenous eosinophil (eosinophil myelocyte
Figures	1		2	3,4,5,6	9	8	
Approximate size	6–9μ	9–12μ	12–15μ	12–30μ	12–30μ	15–35μ	10–18μ
Nucleus	Round or oval. Large in proportion to cytoplasm	Round, oval, or irregular	Round, oval, or indented	Oval usually. Round or indented. Larger than lymphocyte nucleus	One or more. Oval or indented	2–8 or more nuclei around centrosphere	Round or dented
Chromatin	Scattered coarse masses	Scattered coarse masses	Scattered coarse masses	Diffuse reticular network	Diffuse, reticular network	Diffuse, reticular network	Scattered masses
Cytoplasm	Small in amount. Ectoplasm clear and often delicate yellow shade. Endoplasm contains mitochondria	Larger amount but same type as small lymphocyte	Large amount. Same characteristics as small lymphocyte	Hazy. Ground glass-like	Hazy. Ground glass-like	Hazy. Ground glass-like	Clear for granules
Specific granules	None	None	None	None	None	None	Few to yellow, refractive granules
Segregation vacuoles or granules	None to a few	None to a few	None to a few	Few to many very small, brick-red, non-refractive granules grouped in a rosette about the centrosphere	Exceedingly many granules of same type as those in monocyte and in giant cell with rosette	Few to many small, brick-red, non-refractive granules grouped in a rosette about the centrosphere	None
Mitochondria (stain delicately green with Janus green)	Abundant and always perinuclear in endoplasm. Usually round and fairly large in size	Same as in small lymphocyte	Same as in small lymphocyte	Abundant, very small rods, dots, and filaments scattered in cytoplasm around the rosette	Not easily demonstrated because of preponderance of red granules	Abundant and of same type as in monocyte	May be in clear plasmic
Phagocytic properties	Slight if any	Slight if any	Slight if any	Moderately phagocytic	Slight	Slight	None
Where found	In all lymphoid tissue	In superficial and deep lymph nodes and spleen. Abundant in mesenteric nodes	In superficial and deep lymph nodes and spleen. More numerous in mesenteric nodes	Abundant in peripheral nodes. Absent in mesenteric mass of lymph nodes, thymus, and spleen	Never in mesenteric mass, thymus, or spleen. Often in peripheral nodes	Never in mesenteric mass, thymus, or spleen. Often present in peripheral nodes	Often abu dant in racic no Few in terics. number ripheral Rare in mus

Stained Supravitally with Neutral Red.

	Clasmatocyte (macrophage)	Giant cell with one or more nuclei with large, even sized, refractive, cytoplasmic granules	Giant cell with one or more nuclei and cytoplasmic, fusiform bodies together with refractive granules	Large cell containing platelet-like bodies in cytoplasm	Endothelial cells	Primitive cells	Fibroblasts
	7		10	11		12	
	12–30μ	15–35μ	15–50μ	15–50μ	10-20μ Elongated	10–15μ	12–20μ Marked variation
	Oval usually. May be round	One or more oval or indented	Usually eccentric and partially obscured. One or more nuclei. Oval or round	Round or oval. One or more nuclei	Oval	Round or oval	Oval or round
	Diffuse reticular network	Usually diffuse. Often no definite pattern	Scattered, small masses	Scattered, small [illegible]	Diffuse, reticular network	Very indistinct. Not splotchy	Diffuse reticular network
	Filled with vacuoles	Filled with granules	Filled with granules and fusiform bodies	Filled with platelet-like bodies	Often hazy. May be clear	Clear and glassy. Slight yellow tinge	Hazy
	None	The granules or vacuoles are probably degenerative phenomena	Nature of these granules and bodies unknown	This cell is probably a clasmatocyte with ingested platelets	None	None	None
	Filled with uneven, red, brown, and yellow vacuoles varying markedly in size	In a few of these cells granules similar to those in epithelioid cell	Some large and small vacuoles may be present as in clasmatocyte	None seen	May have few non-refractive, brown or yellow granules	None	Occasionally a few small, diffusely scattered, neutral red granules
	Few can be seen in a clear area of cytoplasm if this can be found	Not seen	Not seen	Not seen	May be seen scattered in cytoplasm	Not seen	Few small filamentous bodies
	Marked	Probably not very phagocytic	Probably markedly phagocytic	Probably markedly phagocytic	Moderately phagocytic	None	Little if any
	Abundant in mesenteric nodes and spleen. Few in all lymphoid tissue	Found chiefly in peripheral nodes in small numbers	Never in mesenterics. Often in popliteals, inguinals, and cervicals	Rare. Found occasionally in mesenterics	Found in all lymph nodes	All lymph nodes, spleen, thymus	All organs as units in structural framework

The Lymphocyte.

The living lymphocytes, small, intermediate, and large, have been adequately described and illustrated in the papers of Simpson (11, 13), Sabin (12), and Cunningham, Sabin, and Doan (1).

The only point which can be added to the description of these cells is the presence of a definite ectoplasm and endoplasm in the cytoplasm of the lymphoid cells in the lymph nodes. This characteristic cannot always be seen, but it is likely that it is always present. Usually a definite demarcation can be noted in the nature of a delicate boundary (Fig. 2). The endoplasm is less clear than the ectoplasm, and in films of cells stained with neutral red and Janus green the ectoplasm has a very faint, barely distinguishable, yellow tint. There are no neutral red bodies or mitochondria in the ectoplasm. These structures are within the endoplasmic boundary. The endoplasm may be very narrow, in which case the mitochondria are very near the nucleus, or it may be wide, occupying the major portion of the cytoplasm. It is more common to find the ectoplasm and endoplasm of the lymphoid cells about evenly divided.

The Monocyte.

The cell which has been of particular interest in this study is the monocyte which, as Sabin (12) has shown, is usually characterized by a rosette of neutral red bodies in the cytoplasm. We have consistently found it in the parenchyma of the peripheral lymph nodes, and it has been consistently absent in the mesenteric mass of lymph nodes, in the thymus, and in the spleen. Occasionally one finds a few monocytes in these latter organs, but it is probable that they are cells which have wandered in from the blood and are not parenchymal cells.

With neutral red the rosette cells stain handsomely (Figs. 3 to 6). They are usually large, often occupying three to five times as large an area as the small lymphocyte. The nucleus is large, about twice the size of a small lymphocyte. There is a sharp nuclear membrane. The nucleus is usually oval or round, but may be indented. The chromatin is more scattered and diffuse than in the lymphocyte, forming a reticular network. The cytoplasm may be rounded up, but more often it has an irregular border with delicate, short prolongations into the spaces about the cell. The cytoplasmic border is so delicate that it is frequently difficult to see. Within the cytoplasm one finds usually two types of structures, neutral red bodies and mitochondria.

The great majority of these rosette cells have exceedingly fine granules which barely can be distinguished from one another. These neutral red bodies (segrega-

tion granules) are grouped about a centrosphere often in the center of the cell, or in the bay of the nucleus. Sometimes this group of granules laps over the inner border of the nucleus, obscuring a portion of the nuclear membrane (Fig. 5). The number and quality of these neutral red bodies vary markedly. Some cells have no reaction to the dyes whatever, but present all of the other histological characteristics of the monocyte. It is conceivable that these are young cells which have not reached sufficient maturity for the elaboration of a rosette. Others have a single ring of the segregation granules about the centrosphere (Fig. 3), while some have so many granules and so large a rosette that they resemble the so called epithelioid cells (Fig. 9). Occasionally one finds the entire cytoplasm packed full of these bodies. These segregation bodies stain in exactly the same manner as do the granules of the monocytes of the blood, a brownish red or brick-red color. These bodies are non-refractive. Some of the cells have larger, more discrete granules which are a little more refractive and a little less basophilic, that is, staining slightly more yellow (Fig. 6). All intermediate transitions of these granules can be seen. The mitochondria appear as delicate dots and short, thin rods, which is in contrast to the appearance of the larger and stouter Altmann bodies of the lymphocytes. They stain a delicate bluish green with the Janus green, and are scattered in the cytoplasm, most of them being located near the periphery of the neutral red rosette (Fig. 4). These developing monocytes (rosette cells) are most often found in clumps of several cells (Fig. 13) in various stages of development, some containing a large rosette and one or more nuclei and others, supposedly younger cells, containing no rosette at all or a very small one.

Some, but not all of these monocytes of the lymph nodes are identical with those normally found in the blood. The size of the cells is, on the whole, about the same, or slightly larger than those of the blood. The nucleus is of the same type. A neutral red rosette is present in both types of cells, but the arrangement of the granules about the centrosphere is a more exact and constant feature in the monocyte of the lymph node. The cells in the lymph node do not appear to be actively motile. It seems reasonable to believe that were they transformed into motile cells, as occurs when they become more mature, the arrangement of the granules in the rosette might well be less exact in their position about the centrosphere, making these cells indistinguishable from the monocytes of the blood stream. There is an analogy here with the story, as we know it, of the development of the polymorphonuclear leucocyte. In the bone marrow the myelocytes are non-motile and usually have the specific granules grouped in a rosette about the centrosphere. However, when the myelocyte develops into a motile leucocyte, the rosette is seldom preserved. The mitochondria of the monocytes of the lymph nodes and of the blood are identical in appearance. The monocytes of the peripheral lymph nodes are oxidase-negative, as are the majority of the monocytes of the blood of the rabbit.

The Epithelioid Cell.

The peripheral nodes of some animals show cells presumably of the monocytic strain which have gone beyond the stage of the ordinary

mature monocyte, with a considerable overdevelopment of the neutral red rosette, giving the typical picture of the so called epithelioid cell (Fig. 9). In other respects these cells are identical with the monocyte. Cunningham, Sabin, Sugiyama, and Kindwall (14) have shown, in experiments with the tubercle bacillus, that monocytes may be stimulated to metamorphose into typical epithelioid cells and giant cells of the Langhans type.

Giant Multinucleated Cell with Rosette.

Just as it was found that some cells tended to have an overdevelopment of cytoplasmic structures, it was also true that in other cells the nucleus tended to be multiple. It is not uncommon to find certain of these monocytes with two or more separate, oval nuclei, arranged around a single neutral red rosette (Fig. 8). Except for the increased size and the increased number of nuclei, this cell was in all respects similar to the monocyte. These three types of cells, the monocyte, epithelioid cell, and multinucleated giant cell with rosette were often found side by side in clumps. These cells are undoubtedly closely related.

Histogenous Eosinophil.

Among the cells of the normal lymph nodes of the rabbit, one frequently finds eosinophils with a single or a lobulated nucleus, and coarse, refractive, eosinophilic granules.

These cells usually, but not always, are in clumps. They may be in the lymph cords or on the margins of the lymph follicle. More rarely one finds them in the center of a lymph follicle, tending to crowd out the lymphoid elements. These cells are usually more numerous in the mediastinal lymph nodes, but may be present in considerable numbers in any of the peripheral nodes. There was one case in which the eosinophils were the most conspicuous cells in the whole node. Whether these cells wandered here from the blood stream, or whether they are fixed tissue cells is difficult to answer. The fact that they are often mononuclear and present the appearance of eosinophilic myelocytes leads one to believe that they are probably true histogenous elements, comparable to the histogenous basophils.

Histogenous Basophils.

Another cell which is quite commonly found in lymph nodes of man (Forkner (15)) is also present in all of the lymph nodes of the rabbit.

The histogenous basophil or mast cell occurs sometimes alone and often in groups of two or three cells. It is similar in all respects to the mast cell of the general connective tissues. The cell is large in size and often somewhat irregular in shape, with rounded borders. The nucleus is relatively small and tends to be obscured by the large number of moderate sized refractive basophilic granules which fill the entire cytoplasm. These specific granules of the mast cell stain a brilliant red with the neutral red dye.

The Clasmatocyte.

These cells, variously termed macrophages, histiocytes, pyroll cells, and so forth, have long been known to occupy an important place in the histology of lymph nodes.

They are much more numerous in the mesenteric lymph nodes than in any other group. Here they occur lining the lymph sinuses and sometimes filling almost the entire space occupied by the lymph cords (Fig. 16). They are usually filled with coarsely granular débris. These cells are fragile, and in fresh scrapings most of them are ruptured, liberating a very large amount of free, granular débris. In those cells which are not ruptured, the débris is scattered throughout the cytoplasm and when stained is variously colored with shades of yellow, red, and brown (Fig. 7). The débris also varies markedly in regard to the size of the vacuoles and granules from very minute bodies to structures the size of red blood cells, or larger. In fact, it is not uncommon to find red blood cells themselves engulfed in the cytoplasm. Excellent descriptions and illustrations of clasmatocytes stained with neutral red and Janus green have been given by Simpson (13), Sabin, Doan, and Cunningham (16), and others.

Giant Cell with Even Sized, Large, Refractive Granules.

In an earlier communication, Forkner (15) described a cell found in both normal and pathologic human lymph nodes, which was conspicuous by the presence of one or more nuclei and large, refractive granules which were considerably larger than the specific granules of polymorphonuclear eosinophilic leucocytes, and which did not stain with vital dyes. It was found by Forkner (15) that they occurred commonly in human tuberculous nodes. The present survey of rabbit lymph nodes has revealed the same cell in supravital preparations, but we have been unable to identify it in the fixed tissues. Study of these cells has seemed to indicate quite clearly that they are forms of the

monocytes and epithelioid cells and that the refractive granules probably represent degenerative changes. In some instances monocytes and epithelioid cells were found with varying numbers of these granules, and at the same time they retained traces of the neutral red rosettes. These cells are never numerous.

Giant Cell Containing Cytoplasmic Rods and Fusiform Bodies.

These cells, so far as I know, have never been described. They have been found in these studies in a fairly large proportion of rabbits, always in the peripheral lymph nodes and never in the mesenteric mass of lymphoid tissue.

The cells vary tremendously in size, some of them being as large as the ordinary clasmatocyte and others occupying an entire field of the oil immersion lens (Fig. 10). They are usually in clumps (Fig. 14), but occasionally an isolated cell is seen. The relatively small, oval, or round nuclei may be numerous and are crowded to an eccentric position by the cytoplasmic structures. The chromatin is diffusely scattered. One finds two types of bodies in the cytoplasm. The predominating structures are irregular, fairly even sized, poorly refractive granules which usually do not take the dye but may stain a light yellow or brown. These granules fill the cell to capacity, crowding the nucleus to one side and causing the cytoplasmic borders to be rounded. Among these granules are found varying numbers of long, refractive bodies, scattered indiscriminately in the cytoplasm. These bodies are often curved. They usually appear to be smaller at the ends than in the center, presenting a fusiform effect. They are about 5 microns in length and from 0.2 to 0.5 micron in diameter.

The origin and significance of these cells and their inclusions are not known. At first it was thought that the elongated fusiform bodies might be bacteria. However, the absence of these bodies in other cells, the absence of a polymorphonuclear infiltration, and of other signs of infection speak against this view. Further work is necessary to solve the problem definitely. They may be clasmatocytes, but if so, they are of a different type from the usual macrophage found in the connective tissues generally and in the same lymph nodes. If they are to be called macrophages, they must obey the laws of macrophages with respect to the storing of vital dyes. This test devised by Evans and Scott (17) for connective tissue cells has not yet been tried with respect to these constituents of the lymph nodes.

Large Cell Containing in the Cytoplasm Bodies Resembling the Platelets of the Blood.

There is yet another cell which is described with certain reservations, because it has been observed in the mesenteric nodes of only one animal.

The cells vary considerably in size and shape, and in these respects resemble the clasmatocytes. The nucleus is usually relatively small and is rounded. In the larger cells there may be several nuclei which are near the border of the cell. The points which distinguish this cell from that described in the section immediately preceding are the absence of the fusiform bodies and the presence of irregular, somewhat larger, granular structures in the cytoplasm (Fig. 11). These granular bodies are discrete and contain small stained bodies within their boundaries. They bear a striking resemblance to the platelets of the peripheral blood and may actually be these structures. In the cells where these cytoplasmic inclusions are more numerous and crowded together, they lose their identity, and it is difficult to separate the individual particles. They stain very little with neutral red and only the granules in the platelet-like bodies take the dye.

Endothelial Cells.

It is not uncommon to find ordinary endothelial cells in scrapings of lymph nodes. These come from the endothelium of the blood and lymph vessels. They are elongated cells with a centrally placed oval nucleus. They may have a few neutral red cytoplasmic granules or may remain unstained.

Primitive Cells.

These cells are the most difficult to identify of all the cells found in lymphoid tissue. The cells with which they are likely to be confused are small, intermediate, and large lymphocytes. They differ from these cells, however, in their minute cytoplasmic and nuclear structure.

Their size corresponds to that of the intermediate and large lymphocytes (Fig. 12). Their cytoplasm stains with neutral red to give it a very light yellowish tinge and corresponds very closely to the ectoplasm of the lymphocyte. No endoplasm and no cytoplasmic structures can be identified. In other words, this cell has not yet reached the stage of differentiation with the elaboration of cytoplasmic granules. The nucleus is likewise undifferentiated. It is usually smaller than the nucleus of lymphocytes of corresponding size. The nucleus almost invariably possesses a nucleolus which, in fresh preparations, is somewhat lighter in

shade than the rest of the nucleus. The chromatin of the nucleus is diffuse. There are no clumps or aggregations of chromatin material, which distinguishes this cell sharply from the lymphocyte. This characteristic also makes the nucleus difficult to distinguish from the cytoplasm for they have almost the same refractive power.

These cells were described by Cunningham, Sabin, and Doan (1) in bone marrow which had been reduced to a relatively aplastic condition. They have also been described and illustrated by Kubie (19). He found them along the blood vessels of the brain just outside the endothelial cells. They can be found in considerable numbers in the normal omentum of the rabbit.

Fibroblasts.

Fibroblasts are frequently found in scrapings of lymph nodes. They are part of the structural framework of the nodes.

They are usually elongated, spindle-shaped cells with an oval or round nucleus. The cytoplasm is often irregular, particularly at the ends of the cell. Neutral red and Janus green do not stain them well. Occasionally a few very small, scattered neutral red bodies and delicate, bluish green mitochondria can be made out. They are much fewer in number than the lymphoid cells. An illustration of the fibroblast stained supravitally with neutral red and Janus green is found in a paper by Sabin and Doan (18).

Distribution of Cells in Lymphoid Organs.

The various groups of lymph nodes studied, and the relative numbers of the types of cells found in them are recorded in Table II.

From study of this table, several facts are revealed:

1. The small lymphoid cell is the predominating element in all lymphoid tissue.

2. Intermediate and large lymphoid cells are found in greater numbers in the mesenteric lymph nodes and in those draining the viscera and deeper structures of the body.

3. Monocytes of the type described in this paper are most abundant in the superficial lymph nodes, particularly in the popliteal group, and are completely absent in the mesenteric lymphoid mass, in the thymus, in Peyer's patches, and possibly in the scattered lymphoid tissue of the lung and bone marrow.

4. The epithelioid cells, giant cells with rosettes, and giant cells with refractive granules occur only in those lymph nodes in which monocytes are found.

TABLE II.

Relative Distribution of Various Cell Types in Lymphoid Tissues.

Cells	Popliteal	Flank: Inguinal Pubic: Submaxillary Superficial cervical Superficial axillary Deep axillary	Deep cervical	Mediastinal	Mesenteric	Isolated mesenteric	Retroperitoneal	Thymus	Spleen	Peribronchial lymphoid areas	Peyer's patches
Small lymphoid	++++	++++	+++	+++	+++	+++	++++	++++	+++	++++	++++
Intermediate lymphoid	+	+	++	++	++	++	+	+	+	±	±
Large lymphoid	+	+	+	+	++	++	+	+	+	−	−
Developing monocytes	+++	++	±	±	−	+	+	−	−	?	−
Epithelioid	±	±	±	±	−	±	±	−	−	?	−
Giant cells with rosette	++	+	+	±	−	+	+	−	−	?	−
Eosinophils	+	+	+	++	±	+	+	±	±	±	±
Histogenous basophils	+	+	+	+	+	+	+	±	+	±	++
Clasmatocytes	+	+	+	+	+++	+	+	+	++	++	++
Giant cells with refractive granules	±	±	±	±	−	±	±	−	−	−	−
Giant cells with fusiform bodies	±	±	−	−	−	−	−	−	±	−	−
Giant cells with platelet-like bodies	−	−	−	−	±	−	−	−	−	−	−
Endothelial	+	+	+	+	+	+	+	+	+	+	+
Primitive	+	+	+	+	+	+	+	+	+	?	+
Polymorphonuclear	+	+	±	+	+	+	+	±	+	+	+
Mature red blood cells	±	±	±	±	±	±	±	±	++	+	±

5. Clasmatocytes are present in all lymph nodes but are much more abundant in the mesenteric mass, in the spleen, and in the Peyer's patches.

6. The so called giant cells with fusiform bodies are only found in the superficial nodes and spleen.

7. Giant cells containing platelet-like bodies have been observed only in the mesenteric nodes.

8. Developing red blood cells are not found in the lymphoid tissue of the normal rabbit.

9. Cells with specific eosinophilic granules are found indiscriminately in lymph nodes but are usually much more abundant in the mediastinal group.

It should be mentioned here that studies have been made on the lymph nodes of very young rabbits within the first hours after birth to determine if monocytes were then present or if they appeared during the course of extrauterine development. In these experiments the same relationships have been found in the new-born with regard to the monocytes, as exist in the adult animal.

Correlation of the Supravital Studies with Those on Fixed Tissue.

Lymph nodes in the rabbit vary markedly in size with the color of the animal but their fundamental histology is quite uniform. In our experiments it has usually been the gray rabbit of about 2 to 3 kilograms in weight which possesses the largest nodes.

The Lymph Node Follicle.

The follicle of the lymph node contains small lymphocytes in great abundance, packed closely about the germ center of Flemming (20). Mixed in with these small cells there are many cells with a slightly larger nucleus and somewhat more cytoplasm. These larger cells, however, are much more abundant in the germ center itself. With Giemsa stain many of them have a very dark purple cytoplasm (Fig. 19). Sections stained with hematoxylin and eosin demonstrate them as large, pale cells (Fig. 20). These elements in our interpretation are very early cells from which the lymphocytes develop. They probably correspond to the primitive cell (Fig. 12), as previously described. The nucleus has a diffuse chromatin network and a conspicuous nucleolus. This primitive cell, or so called "germ center cell," is not exclusively found in the germ centers. Cells in every way identical with it are often scattered in the periphery of the follicle and in the lymph cords.

In the germ center one can see cells with hematoxylin and eosin which are apparently transitional in type from the primitive cell with pale, undifferentiated cytoplasm and small nucleolus to the intermediate and large lymphoid cells with basophilic cytoplasm and a larger nucleolus. In this area mitotic figures are more common than anywhere else in the lymph node but they do not occur exclusively here. Dividing cells may also be seen in the cortex of the follicle, in the perifollicular tissue, and even in the medullary portion of the node.

In some cases we have seen tremendous numbers of eosinophils in lymph nodes without any adequate pathological explanation. In one instance the germ center was packed with these cells. Also it has been noticed that in certain superficial nodes some follicles are composed almost exclusively of monocytes (Figs. 15, 17, and 18). These observations are readily interpreted under the view that the primitive cell of the germ center is multipotential.

In sections stained with Giemsa and with hematoxylin and eosin, these rosette cells are much paler than the lymphocytes (Figs. 17 and 18). The nuclei are usually larger and are often indented and elongated. There is usually no nucleolus and the chromatin is small in amount and diffusely scattered. The cytoplasm is very slightly basophilic and contains no stainable granules. These cells are two or three times the size of small lymphocytes. They are not uniformly scattered but tend to be abundant in some areas and scarce in others. They are often found around the periphery of the follicle and sometimes along the endothelium of the lymph sinuses but most of the cells do not appear to have any definite relationship to endothelium.

The Lymph Cords.

The lymph cords are composed chiefly of clasmatocytes, endothelial cells, small, intermediate, and large lymphoid cells. The clasmatocytes contain phagocytic granules which stain green with Giemsa and yellowish brown with hematoxylin and eosin. These cells are usually in close relationship with the sinuses but may be found in areas independent of the lymph channels. In the mesenteric lymph nodes the clasmatocytes or macrophages are found in excessive numbers, almost exclusively in the medullary cords (Fig. 16). They are filled with coarse granular débris. Small, intermediate, and large lymphocytes in all stages of development are found but in much fewer numbers than in the cortex of the node. One also finds occasional primitive cells and in the superficial nodes occasional monocytes in the cords. The other types of cells which were described in the supravital preparations were not distinguishable in the fixed tissues because of the close approximation of the cells and the changes caused by fixation.

Thymus and Spleen.

In this communication no discussion will be given of the histology of the thymus and spleen, but it is noted that the developing mono-

cytes do not occur normally in these organs. Table II shows briefly data concerning the cellular content of these organs. The thymus contains almost exclusively small cells which look like lymphocytes. There are no definite germ centers and very few specific granular cells and clasmatocytes. The spleen contains an abundance of red blood cells, clasmatocytes, primitive cells, and lymphoid cells, chiefly small lymphocytes. In the spleen are also found all of the cellular elements of the blood, including basophils, eosinophils, neutrophils, and monocytes.

Lymph.

This study has included observations on the lymph of the thoracic duct and of the efferent lymphatic vessels of superficial nodes. These superficial nodes were known to contain an abundance of monocytes. The fluids contained almost exclusively lymphocytes, with no greater proportion of monocytes than could be accounted for by contamination with blood. These facts would indicate that monocytes of lymph nodes do not enter the blood stream through the lymphatic vessels, but probably do so by their own motility in much the same manner as the granular leucocytes leave the bone marrow.

DISCUSSION.

The application of the supravital technique to the study of all of the lymph nodes of many rabbits has thrown new light on the much discussed question of the origin and development of the monocyte in this animal. In the circulating blood of the rabbit these cells are present in small numbers (4 to 8 per cent) and because of their relatively small numbers and their obscure origin, the life history of this cell has not been unraveled.

Ehrlich (21, 22) held the view that these cells, which he termed "transitionals," were intermediate types in the development of the polymorphonuclear cells. This view was adequately refuted by the application of the Romanowsky type of stain to blood cells. Schilling-Torgau (23, 24) described the monocyte as making a third strain of white cells of the blood distinct from leucocytes and lymphocytes. Naegeli (25) believes the monocyte to be of myeloid origin. He observed cells of human cases in which the myeloid or lymphoid elements were specifically elevated or depressed, and found that the monocytes followed the myeloid elements in the

fluctuations. Naegeli believes that the origin of monocytes from the lymphatic system was entirely disproved by the work of his pupil Lejeune (26), who found no monocytes in the thoracic duct. This absence of monocytes in the thoracic duct had been demonstrated before by Patella (27), Crescenti (28), and others using fixed tissues for their studies. More recently the supravital technique has been applied to the study of thoracic duct lymph by Simpson (13), Thorne and Evans (29), and by Kindwall (30). They have confirmed the findings of the former investigators. Thorne and Evans (29) concluded that if monocytes are produced in lymph nodes, their rate of production is insignificant. They believed that monocytes and lymphocytes are not closely related cell types.

Kellum and Forkner (31), in studies on experimentally produced leucocytoses in rabbits, observed that the monocytes of the blood behaved independently of the lymphoid and myeloid strains in their fluctuations. Klein (32) agrees with Naegeli that the transitionals and large mononuclears of Ehrlich are derived from myeloblasts. His evidence was based on the study of the blood in cases of chronic myeloblastic leucemia. Pappenheim (33) and Pappenheim and Ferrata (34) believe in a close interrelationship of the monocytes and lymphocytes. Their evidence is based largely on morphological grounds. Pappenheim believes that both monocytes and leucoblasts are derived from the cells which he calls lymphoidocytes. On a morphological basis Bergel (35), Downey and Weidenreich (6), Hirschfeld (36), Maximow (37), and others consider that the monocytes are derived directly from the lymphocytes. Their arguments have been based largely on the character of the granules and nuclear structure. Rhoads and Parker (38) examined lymph nodes of rabbits by the supravital technique, but did not find monocytes. However, with incubation of pieces of spleen, bone marrow, lung, lymph node, and subcutaneous tissue of these animals, they found cells of both the rosette and non-rosette types in the cultures.

Mallory (39, 40) has called attention to the proliferation of endothelium and formation of "endothelial leucocytes" in certain diseases. He identifies these cells with the monocytes of the blood and believes the monocytes are endothelial in origin. McJunkin (2) believes the monocytes have their origin from the "reticular lymph vessel endothelium." His conclusions are based on studies of sections of lymph nodes which had first been injected with neutral red in the living animal. Türk (41) and others are emphatic in their belief that the monocytes arise from the spleen. Cunningham, Sabin, and Doan (1), in material from punctures of the spleen, found from 15 to 20 per cent of cells which they identified as young and adult monocytes. It was their opinion that the monocytes are in large measure derived from the spleen.

Simpson (13) found in animals vitally stained with Niagara blue that the lymph of the thoracic duct always carried a fair number of unmistakable macrophages. Her conclusion was: "It would appear that we cannot have in the lymphatic gland a source of monocyte production and yet this is nevertheless one of the most active sources for macrophage production."

The facts obtained in these studies show that much of the confusion in the study of lymph nodes has been due to normal, but unrecognized histological differences in lymph nodes of different areas of the body. It is no longer possible to draw conclusions from work in which the specific lymph nodes studied are not reported, because of these striking cytological differences. Practically all of the morphological, tissue culture, and other published experimental work on lymph nodes has been recorded without cognizance of this fact. The present studies confirm the findings of Cunningham, Sabin, and Doan (1) that monocytes are not found in the mesenteric nodes. They also confirm the report of McJunkin (2) that monocytes are found in the iliac, inguinal, and axillary nodes. However, the conclusions of McJunkin (2) that monocytes are found in lymph nodes generally are not supported.

In the present studies the spleen and bone marrow have been observed consistently in supravital preparations. Both of these organs undoubtedly contain monocytes, but the typical developing monocytes, such as are found in the superficial lymph nodes, have not been discovered in the spleen. The cells found in the normal spleen in this series of animals were of the adult type encountered in the blood and were not greater in proportion to the other white blood cells than can be accounted for by a filtering out process. This confirms the reports of Naegeli (25) and of Pappenheim and Fukushi (42). From this study it cannot be definitely ascertained whether the bone marrow normally is an active site of formation of monocytes. Cells have been found in this organ which conform closely in morphology to the monocytes of the lymph nodes. These cells, however, are difficult to separate from the early myelocytes. The cells in the marrow which are unquestionably developing monocytes are exceedingly few in number.

In certain pathological states it has been shown by Sabin and Doan (18), by Doan and Sabin (43), and others that the bone marrow, spleen, omentum, mesenteric lymph nodes, and other organs may be the site of active proliferation of monocytes. It seems reasonable to believe that under abnormal conditions the mesenteric lymph nodes, spleen, and other organs where multipotential cells exist might well take over the function of forming monocytes.

I should like also to point out that the relative absence of develop-

ing monocytes, and the great numbers of clasmatocytes in the mesenteric lymph nodes might be due to a purely physiological response. The mesenteric lymph nodes have an intimate relation to the gastro-intestinal tract and are therefore concerned with the products of digestion and of metabolism. This relationship may possibly explain the striking histological differences which exist and which make the mesenteric lymph nodes and the peripheral lymph nodes structurally and functionally two different organs.

SUMMARY AND CONCLUSIONS.

1. Lymph nodes from all parts of the body have been studied in a series of 58 rabbits. All of these studies have been on normal animals or on animals in which there were no specific pathologic lesions of the lymph nodes.

2. The supravital method of studying living cells has been employed together with the method of fixed sections. A correlation of the findings with these two methods is made.

3. Lymph nodes from different parts of the body possess marked differences in their cytology.

4. Developing monocytes have been found as normal constituents of all the lymph nodes of the rabbit except the mesenteric mass lying in the mesentery at the lower border of the stomach. When present the monocytes occur in varying numbers being most abundant in the more superficial nodes, particularly in the popliteal group, where they may constitute one-third to one-half of the total cells present.

5. The position of the developing monocytes in the lymph node is believed to be chiefly in the perifollicular area without definite relationship to the lymph sinuses. In some instances they are also found in the follicle itself or scattered in the medullary area. More accurate knowledge on this point must await further study.

6. During the first hours of life the monocytes are present in the lymph nodes in the same relationship as in the adult animal.

7. The monocytes are not normally present in the efferent lymphatics draining the popliteal nodes and are not normally present in the thoracic duct. It is suggested that the monocytes being extravascular in origin, enter the blood stream by means of their own motility in much the same manner as the granular leucocytes gain ad-

mission to the circulating blood. Unequivocal proof of this point can be obtained only by further investigation.

8. Many of the lymphoid cells of the lymph nodes have shown a definite division of cytoplasm into ectoplasm and endoplasm. The mitochondria and neutral red bodies are sharply limited to the less basophilic endoplasm.

9. Eosinophils are found in great numbers in the mediastinal lymph nodes and are less numerous in the mesenteric and superficial nodes.

10. Clasmatocytes are found in great numbers in the mesenteric lymph nodes where they are concentrated particularly in the medullary areas. They are also present in the spleen. They are much less numerous in other lymphoid tissue.

11. Several cell types not previously described are found to be normal constituents of a large percentage of normal rabbit lymph nodes.

12. Primitive cells are found throughout the lymphoid tissue, but are in greater numbers in the area of the germ center of the follicle.

13. No evidence of erythropoiesis could be found in normal lymph nodes.

14. The spleen and bone marrow in this series of animals have not been normally concerned to any significant degree in the formation and development of monocytes.

15. Further experiments are necessary to demonstrate conclusively whether or not the lymph nodes, other than the mesenteric group, are normally the exclusive source of the monocytes of blood.

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EXPLANATION OF PLATES.

PLATE 21.

FIG. 1. Small lymphoid cell from mesenteric lymph node. Rabbit 164 B. Supravital preparation with neutral red and Janus green. There is a slight yellowish tinge to the cytoplasm. No mitochondria or neutral red bodies are stained.

FIG. 2. Large lymphoid cell from mesenteric lymph node. Rabbit 164 B. Neutral red and Janus green stain. Note ectoplasm and endoplasm. Mitochondria are stained bluish green and are limited to endoplasm. Endoplasm is hazy in appearance, whereas ectoplasm is clear and glassy.

FIG. 3. Young monocyte from inguinal lymph node of Rabbit 27 B. Neutral red stain. Cell has two nuclei and one small rosette of segregation bodies around the centrosphere. Note hazy appearance of the cytoplasm.

FIG. 4. Monocyte from popliteal lymph node of Rabbit R 621. Neutral red and Janus green stain. Indented nucleus and neutral red rosette. Mitochondria around periphery of rosette, stained with Janus green.

FIG. 5. Same as Fig. 4. Oval nucleus with rosette overlapping one border of nucleus. Three small, refractive vacuoles, unstained, are beginning to develop in cytoplasm.

FIG. 6. Monocyte from popliteal node of Rabbit 92 B. Neutral red stain. Segregation granules are larger and are yellowish brown in color. This cell is closely packed, causing the approximately rectangular shape.

FIG. 7. Clasmatocyte (macrophage) from mesenteric lymph node of Rabbit 92 B. Neutral red stain. Notice the irregularity in size and shape of vacuoles which are arranged in no definite pattern in cytoplasm. Note also the different colors of the different vacuoles.

FIG. 8. Multinucleated giant cell with a single rosette from superficial axillary lymph node of Rabbit 32 B. Neutral red stain. These cells may possess from one to several nuclei.

FIG. 9. Epithelioid cell from retroperitoneal lymph node of Rabbit 32 B. Neutral red stain. This cell is very similar to the monocytes of lymph nodes. It represents the same strain of cells. It is probably a monocyte with a hypertrophied rosette.

FIG. 10. Giant cell containing many irregular granules and numerous elongated rod- or sickle-shaped bodies in cytoplasm. From popliteal lymph node of Rabbit 38 B. These cells may have one or several nuclei. Cytoplasmic structures stain only with heavy concentration of dye.

FIG. 11. Giant cell with bodies like blood platelets in cytoplasm. These were found in only one animal.

FIG. 12. Primitive cell from spleen of Rabbit 53 B. Neutral red stain. Notice relatively little differentiation of cell. Nucleus has large nucleolus. No cytoplasmic structures could be discerned. A small lymphocyte has been sketched for comparison of size.

PLATE 22.

FIG. 13. Group of monocytes and epithelioid cells from popliteal lymph node of Rabbit 51 B. Magnification × 1000. A few lymphocytes can be seen in the photograph. Cells stained with neutral red. Notice the heavy rosettes of neutral red granules.

FIG. 14. Clumps of giant cells containing granules and rod- or sickle-shaped bodies in cytoplasm. Supravital film with faint neutral red dye. Magnification × 1000. One of these cells was drawn and is illustrated in Fig. 10. Notice lymphocytes near the giant cells.

PLATE 23.

FIG. 15. Two follicles from superficial axillary lymph node of Rabbit R 452. Hematoxylin and eosin. Magnification × 450. Note the follicle on the right. It is composed almost exclusively of monocytes which are very much larger and paler than the lymphocytes of the other follicle.

FIG. 16. Mesenteric lymph node of Rabbit R 452. Magnification × 450. Giemsa stain. The node is not homogeneous. The cortex (left side of figure) contains almost exclusively lymphoid cells. The sinuses and lymph cords are crowded with clasmatocytes (macrophages). These latter cells are shown lining the sinuses and scattered in the parenchyma of the node. They contain numerous vacuoles in their cytoplasm.

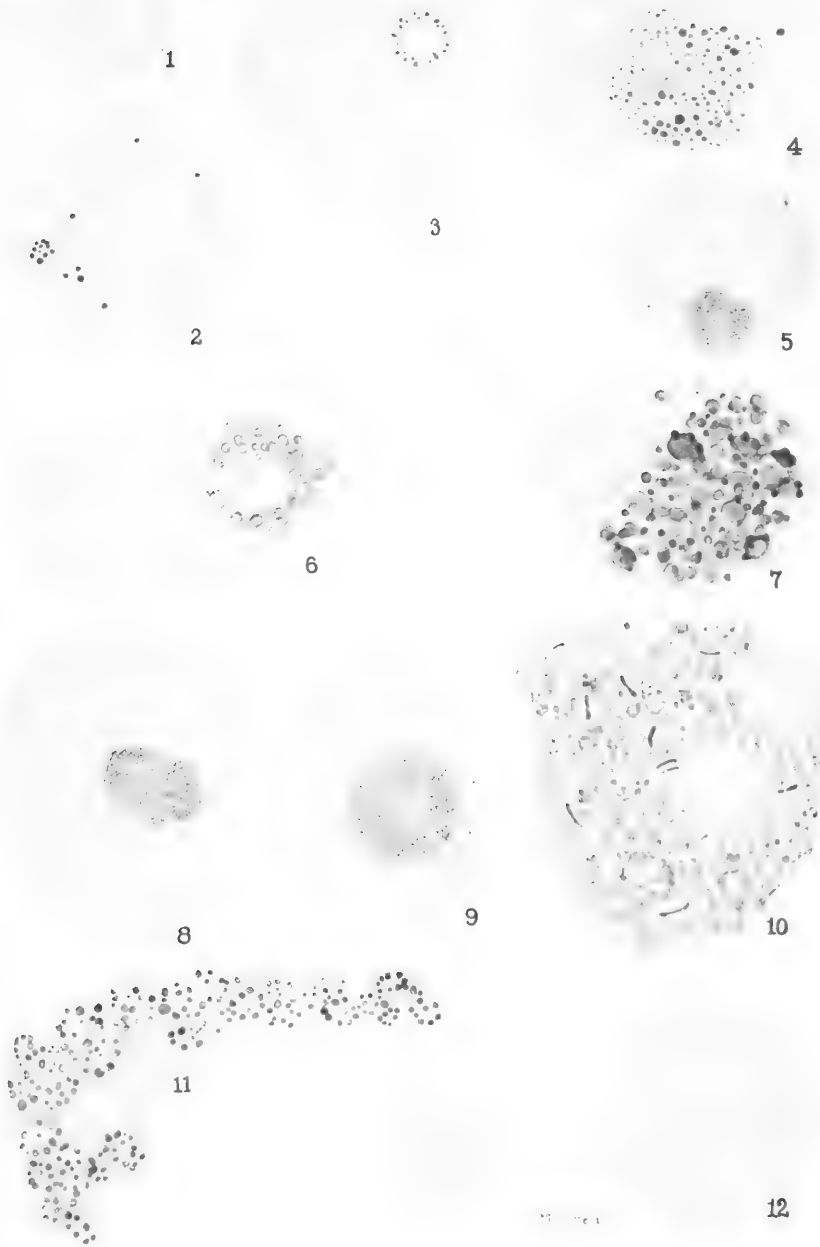
PLATE 24.

FIG. 17. Higher magnification of the follicle on the right of Fig. 15. A follicle composed almost exclusively of monocytes. Giemsa stain. Magnification × 1000. Note the large, pale monocytes in contrast to the heavily stained small lymphocytes.

FIG. 18. Same as Fig. 17. Hematoxylin and eosin stain. The character of the nuclei of the monocytes can be more clearly seen than with Giemsa stain. The monocytes show much less basophilia than germ center cells or young lymphocytes.

FIG. 19. Germ center cells of pelvic lymph node. Rabbit R 452. Giemsa stain. Magnification × 1000. Note particularly the very heavily stained, basophilic cytoplasm of the large germ center cells.

FIG. 20. Same as Fig. 19. Hematoxylin and eosin stain. Note the paleness of the cells with this stain. Contrast germ center cells of Figs. 19 and 20 with the monocytes of Figs. 17 and 18.



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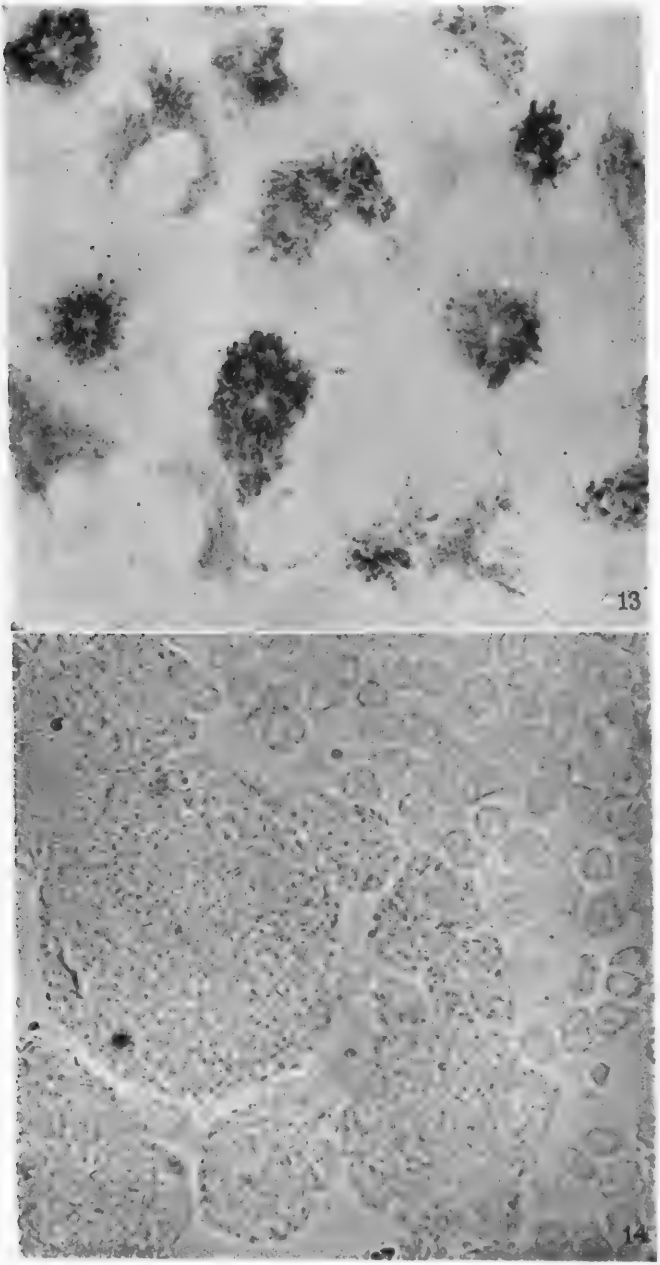

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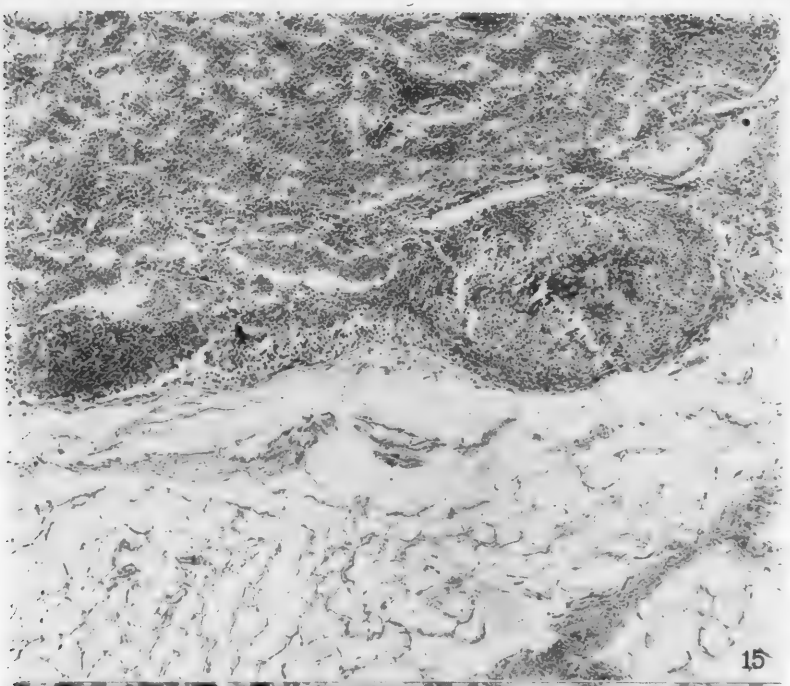

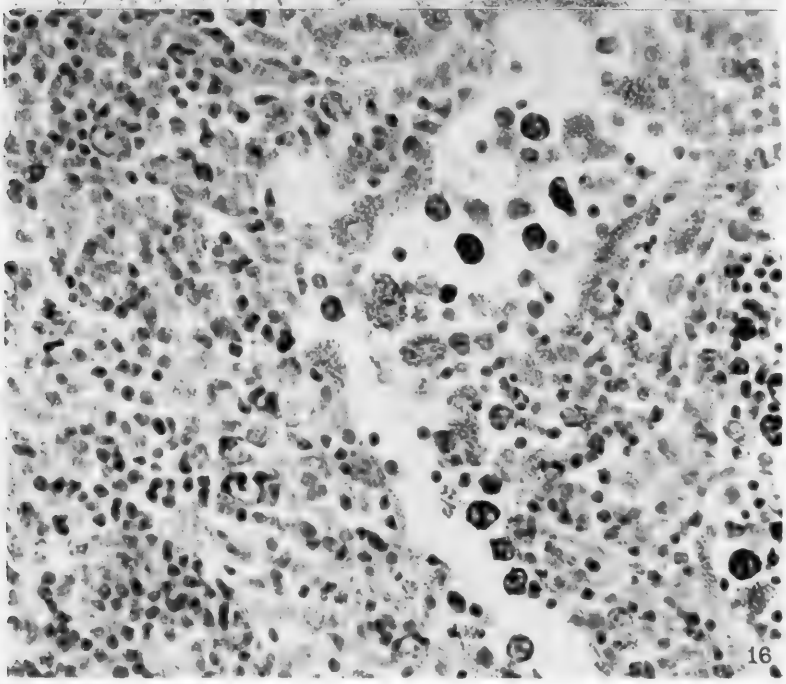

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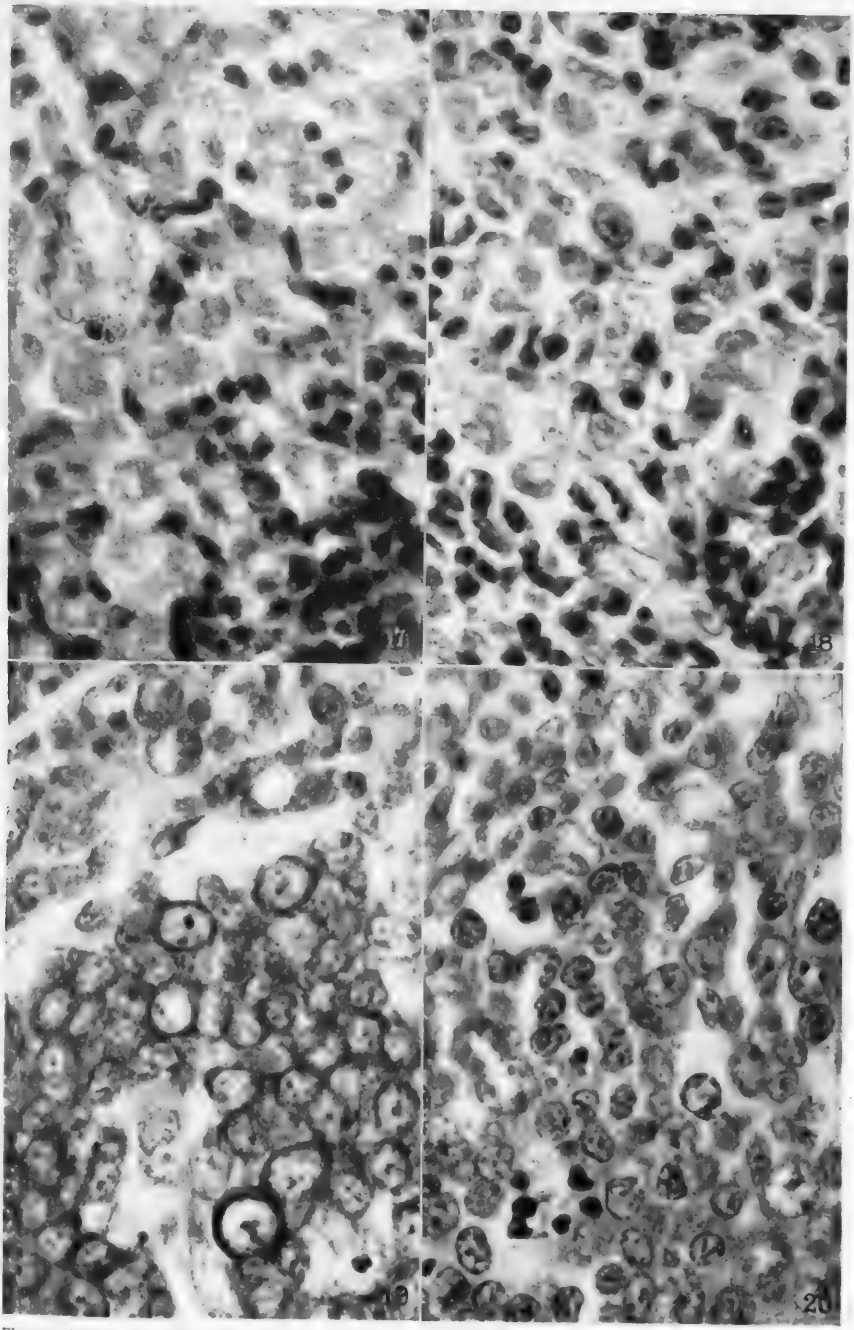

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